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STUDY OF THE FUNCTION OF PYRIDOXAL 5'-PHOSPHATE IN GLYCOGEN PHOSPHORYLASE

Iowa State University PH.D. 1984

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Study of the function of pyridoxal 5'-phosphate in glycogen phosphorylase

by

Yen-Chung Chang

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1984

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DEDICATION

To my aunt and uncle -

ABBREVIATIONS

AMP	Adenosine 5'-monophosphate
AMP-S	Adenosine 5'-thiophosphate
EDTA	Ethylenediamine tetraacetate
glucose-1-P	Glucose-1-phosphate
P_i	Inorganic phosphate
ppm	Parts per million
PLP	Pyridoxal 5'-phosphate
6-FPLP	6-Fluoropyridoxal 5'-phosphate
6-FPAL	6-Fluoropyridoxal
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser Effect

GENERAL INTRODUCTION

Glycogen phosphorylase has an important catalytic and regulatory role in glycogen metabolism. In vitro, the enzyme, purified from rabbit skeletal muscle, can reversibly catalyze the following reaction:

$$G_{n+1} + P_i \rightleftarrows G_n + G\text{-}1\text{-}P$$

where G_{n+1} and G_n represent glycogen molecules containing n+1 and n sugar moieties, respectively; P_i is inorganic phosphate and G-1-P is glucose-1-phosphate. The equilibrium constant of this reaction at pH 6.8, $(G\text{-}1\text{-}P)/(P_i)$, has been measured to be 0.28 (1,2). However, there is convincing evidence indicating that the enzyme functions in the direction of the cleavage of glycogen in vivo because the ratio of P_i and G-1-P concentration greatly exceeds the equilibrium constant (3).

The activity of phosphorylase can be regulated by the covalent modification of the enzyme (phosphorylation-dephosphorylation) and by ligand-induced conformational changes. The phosphorylated form of phosphorylase, having a phosphate group on Ser-14, is named phosphorylase a, and the protein without this phosphate group is named phosphorylase b. The interconversion between these two forms is controlled by phosphorylase kinase (4) and by phosphorylase phosphatase (5),

as shown in Figure 1.

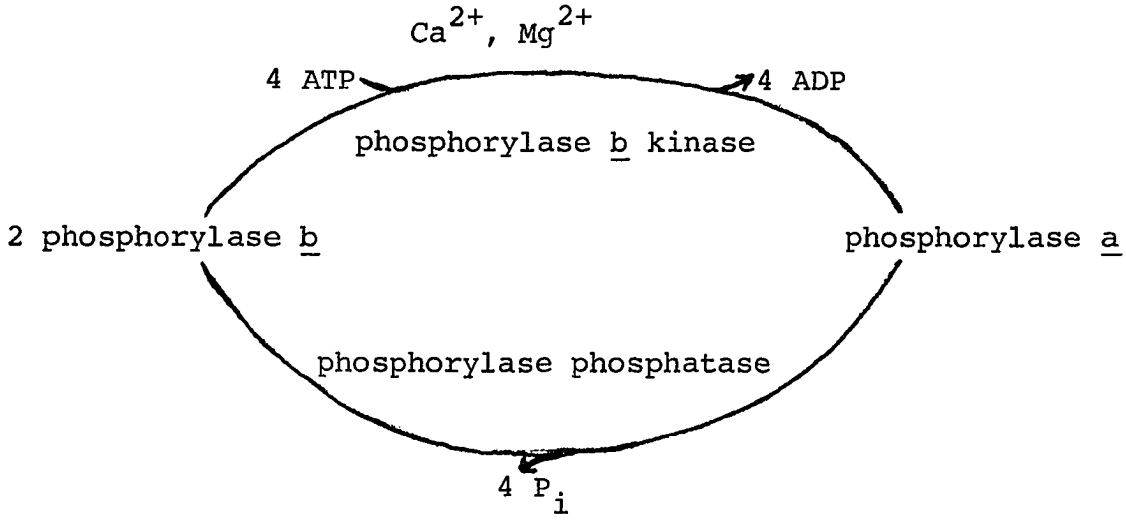

Figure 1. The interconversion of phosphorylase a and phosphorylase b

The nonphosphorylated phosphorylase b shows catalytic activity only in the presence of AMP, while phosphorylase a is active itself. Other different properties of these two proteins are summarized in Table 1.

Table 1. Properties of phosphorylase a and phosphorylase b

Phosphorylase b	Phosphorylase a
1. Inhibited by glucose-6-P and ATP	1. Not inhibited by ATP or glucose-6-P
2. Binds substrates cooperatively	2. Binds substrates without cooperativity
3. Dimer, MW = 200,000	3. Undergoes dimer ⇄ tetramer formation

Phosphorylase is an oligomeric enzyme (6) which consists of identical subunits. In solution, phosphorylase b exists in an equilibrium between a tetrameric and a dimeric species. Because the dimer-dimer interaction in phosphorylase b is very weak, it is mainly in a dimeric form (1). Formation of tetramer can be induced by the obligatory activator of phosphorylase b, AMP (7). And the AMP-induced association can be further enhanced by a number of ionic species; Mg^{2+} (8), F^- (9), polyamines (10), and the substrates P_i and glucose-1-P (11). On the other hand, polysaccharides (12, 13), glucose, caffeine, and glucose-6-P (14, 15) were shown to prevent association of phosphorylase b by AMP. Various results have shown that the dimer of the b form is the catalytically active species of phosphorylase (12, 13). The study of association-dissociation phenomena of phosphorylase b also indicates that the binding of varied ligands can induce certain local conformational changes which can, in turn, influence the quaternary structure of the enzyme. Results of kinetic studies suggest that the binding of varied ligands to their specific sites on the protein can induce changes of the protein structure and hence affect the enzymatic activity. AMP is found to bind to phosphorylase cooperatively, and the activation of phosphorylase b by AMP is the first example of an allosteric effect on an enzyme. Kinetic and equilibrium

binding studies have also revealed various interactions among ligand-binding sites of phosphorylase b. The following table summarizes the important allosteric interactions, Table 2. X-ray crystallographic studies showed that the binding of a ligand to its specific site on protein can cause certain changes of the protein structure. When glucose was replaced by glucose-1-P in phosphorylase a crystals, pronounced

Table 2. Allosteric interactions in rabbit muscle phosphorylase b[a]

Homotropic interaction ligand	Effect[b] of other ligands (heterotropic interactions)			
	Positive effectors		Negative effectors	
	Affecting affinity	Affecting homotropic interaction	Affecting affinity	Affecting homotropic interaction
AMP	P_i, G-1-P glycogen	P_i	G-6-P ATP	ATP G-6-P
G-1-P	AMP	AMP	ATP G-6-P	ATP G-6-P
P_i	AMP	AMP	ATP G-6-P	ATP G-6-P

[a]Reprinted from D. J. Graves and J. W. Wang (1).

[b]The effect is directed toward the ligands in the first column.

structural changes around the N-terminal region were observed (16, 17). And, when the glucose-1-P binding site was occupied by glucose cyclic 1,2-phosphate, which has been suggested to stabilize the enzyme in a more active conformation, substantial structural differences from the "T" state conformation of phosphorylase were also detected (18). Results obtained with both kinetic and physical method studies indicate that phosphorylase, either in solution or in crystalline form, exists in at least two states, "T" and "R" states.

Glycogen phosphorylase contains stoichiometric amount of pyridoxal phosphate (PLP) (19). This prosthetic group is covalently bound to the protein by a Schiff's base with Lys-679 (20). Unlike the rest of PLP-containing enzymes, when the Schiff's base in phosphorylase is reduced by $NaHB_4$, the resulting enzyme still retains more than 60% of the original activity (21). This observation indicates the uniqueness of the function played by PLP in phosphorylase. Although the details of the function of this coenzyme in phosphorylase have not been elucidated, various studies showed that PLP is an indispensable constituent for both the structural integrity and the enzymatic activity of phosphorylase.

That PLP is a structural determinant of phosphorylase has been demonstrated by the fact that apophosphorylase a and b are less stable than the native proteins (21, 22) and that the quaternary structures of apophosphorylase a and b are quite different from those of the native enzymes (23, 24).

Because the removal of PLP results in an apophosphorylase showing no catalytic activity, this result indicates that the coenzyme is essential for catalysis (25). In order to investigate the possibility that a certain functional group of the enzyme-bound PLP may participate in catalysis, different PLP analogues, chemically modified at varied positions of the molecule, have been tested for their effects on apophosphorylase (1, 26). Because any modification at the 4'-position resulted in compounds that did not bind to the apoenzyme, it indicates that the free aldehyde group is essential for PLP binding (21, 27). Phosphorylase reconstituted with PLP modified at the 3-position, O-methyl pyridoxal phosphate (27), and the 1-position, N-methyl pyridoxal phosphate (28) and pyridoxal phosphate N-oxide (29), have been also studied. Phosphorylase reconstituted with O-methyl pyridoxal phosphate was active, suggesting that the dissociable proton of the phenolic group is not involved in a proton shuttle necessary for enzymatic activity. N-methyl pyridoxal phosphate does not bind to apoenzyme; pyridoxal

phosphate N-oxide binds to apophosphorylase but is gradually converted to PLP when it is bound to the enzyme. The N-oxide analogue of 3-methyl pyridoxal phosphate binds to the apoenzyme weakly, and the reconstituted enzyme is inactive (30). However, the steric effect due to the presence of oxygen atom and methyl group in the coenzyme could be introduced in the resulting enzyme and make the interpretation of these results rather complicated. Thus, the function of the ring nitrogen of PLP in phosphorylase is still unclear. Phosphorylase reconstituted with PLP analogues modified at the 2- and 6-positions exhibit diminished activities and thus these positions were eliminated as being essential for catalysis (27, 31). Most recent attention has focused on the 5'-phosphoryl group of the coenzyme and its potential involvement in catalysis. Most modifications of the 5-position yield inactive enzymes, when a) the 5'-phosphoryl group is missing, b) the tetrahedral conformation of the 5'-phosphoryl group has been altered, or c) the second protonable group of phosphate is blocked (1, 26). Parrish et al. (32) showed that pyridoxal reconstituted phosphorylase, which is completely inactive, can partially recover its enzymatic activity in the presence of non-covalently bound phosphate or various phosphate analogues. Therefore, it is likely that the 5'-phosphoryl group of

PLP may somehow participate in the catalytic process of phosphorylase.

Another important role of PLP phosphate in phosphorylase has been suggested by Yan et al. (33). These authors found that the dephosphorylation reaction of native phosphorylase a, catalyzed by phosphorylase phosphatase, could be activated by caffeine or glucose. However, in phosphorylase a reconstituted with pyridoxal, neither glucose nor caffeine could activate this reaction unless the site where PLP phosphate resides in the native enzyme was occupied by phosphate or phosphate analogues. This observation suggested that PLP phosphate in phosphorylase is also important in communicating the structural changes at different parts on the enzyme.

The 3-D structures of phosphorylase a and b were determined by X-ray crystallographic studies in two laboratories (34, 35). The Ser-14-phosphate and the binding sites of PLP, glucose-1-P, glycogen, AMP, and caffeine on the protein were thus located. It has been shown that the enzyme-bound PLP resides next to the active site where the substrate, glucose-1-P, binds, and the closest distance between the phosphoryl groups of PLP and glucose-1-P is only 5.3Å (36). PLP is buried in a rather hydrophobic environment, and the 5'-phosphoryl group is the only functional group of the coenzyme

accessible to the solvent. In addition to Lys 679, which is covalently linked to PLP, Asn 677, Gly 676, Thr 675, and Phe 680 are also close enough to interact with the coenzyme. The carboxyl group of Glu 645 makes a close contact with the pyridine ring nitrogen while the ε-N of Lys 654 is associated with the phenolic oxygen. Several positive charges, Lys 573, Lys 667, and Arg 568, are near or potentially available to neutralize the phosphate group of PLP. Furthermore, the phosphate moiety is found near the N-terminal end of the same α-helice to which the PLP is bound, and may be stabilized by the helix dipole. Among the amino acid residues around the PLP binding site, the function of Arg 568 seems to be exceptionally interesting. Chemical modification study of phosphorylase indicated that an arginine residue in the active site is important to the catalysis (37). On the basis of the kinetic study of phosphorylase with varied arginine analogues, it was found that analogues with lower pK_a values showed greater inhibitory effects (38). Phenyl glyoxal, a compound reacting preferentially with the quanidino groups with lower pK_a value (39), can interact specifically with Arg 568 in phosphorylase and irreversibly inhibit the enzyme (40). These observations suggested that the guanidino group of Arg 568 may have exceptionally low pK_a and that it is important to catalysis.

Because the 5'-phosphoryl group of PLP is in close proximity of the enzyme-bound substrate and is essential for the catalysis of phosphorylase, several mechanisms of the role of PLP, especially the phosphate group, in phosphorylase have been proposed by different groups. On the basis of ^{31}P NMR studies of phosphorylase, the 5'-phosphoryl group was proposed to act as a proton donor during catalysis (41, 42). ^{31}P NMR spectrum of free phosphorylase b showed a resonance at -0.2 ppm from a phosphoric acid (85%) external standard; the presence of AMP-S and arsenate shifted this signal to -3.6 ppm. The spectrum of phosphorylase a showed a major peak at -3.6 ppm and a minor peak at -0.2 ppm, and the smaller peak at -0.2 ppm disappeared when AMP-S was included in the enzyme solution. According to the pH dependency of the ^{31}P NMR chemical shift of PLP, the peak at -3.6 ppm was assigned to a dianionic PLP phosphate, and the peak at -0.2 ppm was assigned to a monanionic species. Because the enzyme showing a -3.6 ppm ^{31}P NMR signal is more active than that with a -0.2 ppm signal, it was concluded that the PLP phosphate donates a proton when free phosphorylase is transformed to an activated state. Similar observations were obtained with potato phosphorylase (43) and E. coli maldextrin phosphorylase (44). Studies with pyridoxal phosphate monomethyl ester reconstituted phosphorylase (30) and with

α-glucal (45) are also consistent with this hypothesis. However, the study of phosphorylase reconstituted with pyridoxal did not support this mechanism. It was found that fluorophosphate can activate pyridoxal enzyme to the same extent as phosphite can and the pH profiles of enzymatic activity of these two anions are almost identical. However, the pK_a value of fluorophosphate is 4.8, and this anion is quite unlikely to donate or accept a proton at the reaction condition, pH 6.8 (46). An alternative explanation of the change of the ^{31}P NMR chemical shifts of active and inactive phosphorylases is that the bond angle between oxygens of the phosphoryl group, O-P-O, which is known to influence the chemical shift of the central phosphorus atom (47), is different in active and inactive proteins.

Another hypothesis was proposed by Johnson et al. (36) in which the dianionic PLP phosphate acts as a nucleophile which can stabilize the carbonium intermediate of glucose-1-P during catalysis. Uhing et al. (48) found that adding organic solvent in phosphorylase solution can increase enzymatic activity, and this observation was interpreted that the presence of organic solvent can reduce the dielectric constant of the solution and thus strengthen the interaction between dianionic PLP phosphate and carbonium intermediate. However, Takagi et al. (49) found that phosphorylase reconstituted with pyridoxal (5') phospho (1)-β-D-glucose showed

no enzymatic activity even after prolonged incubation with substrates and activator. This finding was inconsistent with the participation of the phosphate group of the coenzyme in a covalent glucosyl-PLP intermediate.

On the basis of studies with phosphorylase reconstituted with pyridoxal(5')diphospho(1)-α-D-glucose, Withers et al. (50) proposed an interesting mechanism in which the coenzyme phosphate acts as an electrophile and forms a trigonal bipyramidal configuration. The formation of this intermediate can produce some inductive effect on the substrate and thereby labilize the glycosidic bond. In support of this mechanism, Takagi et al. (51) showed that radioactive glucose from pyridoxal(5')diphospho(1)-α-D-glucose could be incorporated into the nonreducing ends of polysaccharide. Study with pyridoxal pyrophosphate reconstituted phosphorylase showed that this enzyme mimics the native enzyme in the "R" state, and this result also indicated that the phosphoryl groups of glucose-1-P and PLP may interact directly during catalysis (52).

Although the PLP in phosphorylase has been studied extensively, its function, however, still remains a mystery. The main goal of my dissertation work is to seek the answers of the following questions about the function of PLP in rabbit muscle glycogen phosphorylase. 1) Does the PLP

phosphate affect the binding of the substrate, glucose-1-P? 2) What are the properties that are needed for the PLP phosphate to activate phosphorylase? 3) What is the function of PLP during the structural transformation of phosphorylase from an inactive state to an activated state? 4) Are the 3-phenolic group and the ring nitrogen of PLP involved in any acid-base reaction essential for catalysis? Phosphorylase reconstituted with pyridoxal, 6-fluoropyridoxal, and 6-fluoropyridoxal phosphate were employed in this study in order to solve these questions.

Explanation of Dissertation Format

This dissertation follows an alternative format in which there are three sections after the General Introduction. The first section mainly concerns the first two questions mentioned previously, and has been published in Biochemistry (53). The other two sections concern the last two questions and will be submitted into the Journal of Biological Chemistry. Finally, a general discussion of the dissertation, an outlook for future experimentations, and my personal speculation of the function of PLP in phosphorylase are included.

SECTION 1: FUNCTIONS OF 5'-PHOSPHORYL GROUP OF PYRIDOXAL 5'-PHOSPHATE IN PHOSPHORYLASE: A STUDY USING PYRIDOXAL-RECONSTITUTED ENZYME AS A MODEL SYSTEM†

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ABSTRACT

Pyridoxal-reconstituted phosphorylase was used as a model system to study the possible functions of the 5'-phosphoryl group of pyridoxal 5'-phosphate (PLP) in rabbit muscle glycogen phosphorylase. Kinetic studies were conducted by using competitive inhibitors of phosphite, an activator, and α-D-glucopyranose 1-phosphate (glucose-1-P) to study the relationship between the PLP phosphate and the binding of glucose-1-P to phosphorylase. Fluorine-19 nuclear magnetic resonance (^{19}F NMR) spectroscopy of fluorophosphate bound to pyridoxal phosphorylase showed that its ionization state did not change during enzymatic catalysis. Evaluation of the apparent kinetic parameters for the activation of pyridoxal phosphorylase with different analogues having varied pK_{a2} values demonstrated a dependency of K_M on pK_{a2}. Molybdate, capable of binding as chelates in a trigonal-bipyramidal configuration, was tested for its inhibitory property with pyridoxal phosphorylase. On the basis of the results in this study, several conclusions may be drawn: (1) The bound phosphite in pyridoxal phosphorylase and possibly, the 5'-phosphoryl group of PLP in native phosphorylase do not affect the glucose-1-P binding. (2) One likely function of the 5'-phosphoryl group of PLP in native phosphorylase is acting as an anchoring point to hold the PLP molecule and/or

various amino acid side chains in a proper orientation for effective catalysis. (3) The force between the PLP phosphate and its binding site in phosphorylase is mainly electrostatic; a change of ionization state during catalysis is unlikely. (4) Properties of the central atoms of different anions are important for their effects as either activators or inhibitors of pyridoxal phosphorylase. (5) Our results with molybdate are consistent but do not prove that a trigonal-bipyramidal structure of PLP is involved in the catalytic mechanism of phosphorylase.

INTRODUCTION

Various studies of α-glucan phosphorylases reconstituted with vitamin B_6 analogues suggest that the 5'-phosphoryl group of PLP is likely to be involved in the catalytic process [see reviews by Helmreich & Klein (1) and Graves & Wang (2)]. Although phosphorylase reconstituted with pyridoxal is inactive (3), it was found some phosphate analogues could activate the enzyme (4). These findings were explained by binding of phosphate and analogues at the site where the 5'-phosphoryl group of the coenzyme in native phosphorylase resides. Inorganic pyrophosphate was found a potent inhibitor to pyridoxal-reconstituted phosphorylase and was competitive with both the activator, phosphite, and the substrate, glucose-1-P. One mole of pyrophosphate bound per mole of pyridoxal phosphorylase. These findings suggested that the glucose-1-P binding site and the binding site of the 5'-phosphoryl group of PLP are in close proximity and the inhibition by pyrophosphate could occur by extending across the two sites (4). The proximity between coenzyme and glucose-1-P has been firmly established by high-resolution X-ray crystallographic maps developed from two laboratories (5,6).

Several attractive mechanisms have been proposed for the involvement of the coenzyme phosphate in the catalytic

mechanism. Johnson et al. (7) suggested that the coenzyme phosphate may function as a dianion to carry out a nucleophilic attack on the C-1 carbon of glucose-1-P. From the studies of the effects of 1,2-dimethoxyethane on phosphorylase, coenzyme phosphate was suggested to be able to stabilize the glucosyl cation in the intermediate (8). From extensive ^{31}P NMR studies, an alternative mechanism was suggested in which the coenzyme dianionic phosphate acts as a proton acceptor-donor during the glucosyl-transfer reaction (1, 9, 10). Recent findings of Palm et al. (11) with α-D-glucal and Klein et al. (12) with α-D-glucal are consistent with this proposed mechanism. On the basis of studies with phosphorylase reconstituted with pyridoxal(5')diphospho(1)-α-D-glucose, Withers et al. (13) proposed an interesting mechanism in which the coenzyme phosphate could act as an electrophile, which would produce some inductive effect on the substrate and thereby labilize the glucosidic bond. In support of this mechanism, Takagi et al. (14) showed that radioactive glucose from pyridoxal(5')-diphospho(1)-α-D-glucose could be incorporated into the nonreducing end of glycogen. The possibility that a direct interaction of the substrate and the coenzyme phosphate occurs is supported by the recent results of Withers et al. (15) that showed the conformational state of phosphorylase reconstituted with pyridoxal pyrophosphate mimics the active state found in the native phorphorylase.

To gain a better understanding of the characteristics of the 5'-phosphoryl group of PLP in the native enzyme, further studies were done on phosphorylase reconstituted with pyridoxal. The use of this enzyme form allowed us to assess the influence of anions, varying in size, geometry, ionization state, and electronic configuration, bound to the phosphoryl site on catalysis and to evaluate the role of the coenzyme phosphoryl group in the binding of substrate, glucose-1-P.

MATERIALS AND METHODS

Rabbit skeletal muscle was obtained from Pel-Freeze Co. [U-^{14}C]Glucose-1-P was purchased from Amersham. Methylphosphonic acid and ethylphosphonic acid were obtained from Alfa Products. (Aminomethyl)phosphonic acid and glucose-1-P were obtained from Sigma Chemical Co. Purified disodium fluorophosphate, a generous gift of Dr. S. C. Yan, was prepared following a modified method of Higgins and Baldwin (16) as described by Yan and Graves (17). Glucose cyclic 1,2-phosphate was synthesized from glucose-1-P according to the method of Zmudzka and Shugar (18) modified by a procedure described by Dreyfus et al. (19). All other materials were the highest quality commercially available.

Rabbit skeletal muscle glycogen phosphorylase was prepared according to Fischer and Krebs (20) except that 30 mM 2-mercaptoethanol was substituted by cysteine. Apophosphorylase b was prepared by the method of Graves et al. (21). No enzymatic activity could be detected from the apophosphorylase used in this study, even at high protein concentration (0.2 mg/mL). After incubation of apoenzyme (1 mg/mL) with a 10-fold excess of pyridoxal 5'-phosphate for 30 min at 30°C, the resulting enzyme showed a specific activity of 40 μmol/(min.mg). Apophosphorylase a was prepared by the method of Uhing et al. (8). Pyridoxal-reconstituted phosphorylase

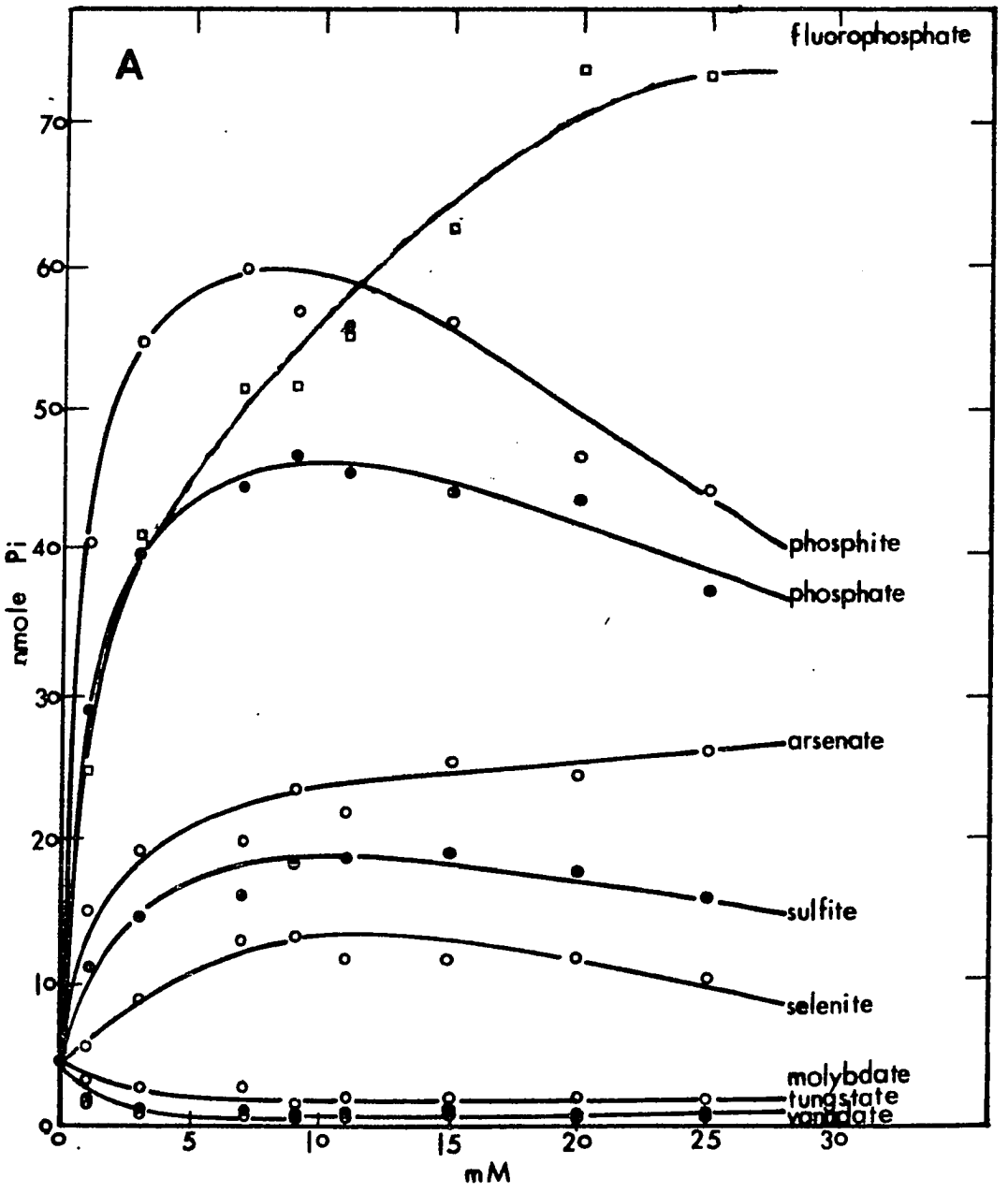
was prepared by the method of Parrish et al. (4). Enzymatic activity in the direction of glycogen synthesis was measured either by liberation of inorganic phosphate from glucose-1-P described by Illingworth and Cori (22) or by the incorporation of [^{14}C]glucose into glycogen with the filter paper assay of Thomas et al. (23). When the activity was measured in the presence of anions, the conditions used are indicated in the appropriate figure legends. The protein concentration of phosphorylase was measured spectrophotometrically by using the extinction coefficient $E^{1\%}_{10mm}$ at 280 nm of 13.2, according to Kastenschmidt et al. (24).

^{19}F NMR spectra were obtained at 282.4 MHz on a Bruker MW300 superconducting spectrometer operating at 20°C. A spectral width of 10000 Hz was employzed with a 20-μs pulse width and a repetition time of 1 s. Exponential line broadening used before Fourier transformation was 5 Hz. Ten percent D_2O(v/v) was present in the buffer used for field/frequency lock, and a 1-mm tube containing TFA (trifluoroacetic acid) was inserted for chemical shift referencing.

RESULTS AND DISCUSSION

To determine the properties of anions necessary to activate pyridoxal phosphorylase, the effects of various anions on the enzymatic activity of the pyridoxal-reconstituted phosphorylase were studied. Because the effect of some of these ions had been reported earlier at one concentration (4), studies were extended to evaluate effects at different concentrations (Figure 1), to evaluate kinetic parameters of activation, and to provide a basis for comparison for other ions not tested earlier. Pyridoxal phosphorylase a was used along with pyridoxal phosphorylase b because it is known that the phosphoryl group in the a form has a stabilizing effect on the pyridoxal 5'-phosphate binding site (25). The results with pyridoxal phosphorylase b (Figure 1A) show that phosphate, phosphite, fluorophosphate, arsenate, sulfite, and selenite can reactivate the enzyme to different extents, whereas, arsenite, sulfate, bicarbonate, nitrate, and nitrite from 0.1 to 5 mM showed no effect (not illustrated). Other anions, ClO_3^-, ClO_4^-, CrO_4^{2-}, $H_2BO_3^-$, F^-, and the divalent cations Mg^{2+} and Mn^{2+}, at 5 mM, had no effect. A similar effect of anions on pyridoxal phosphorylase a was found (Figure 1B) except it seems that less activation was afforded by selenite in this case. No activation of native phosphorylase b or a could be observed by these activators, showing that activa-

Figure 1. Effect of anions on enzymatic activity of pyridoxal reconstituted phosphorylase. (A) The assay solutions at pH 6.8 and 30°C contained pyridoxal-reconstituted phosphorylase *b* (78 µg/mL), 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.02 M β-glycerophosphate, 0.016 M [U-^{14}C]-glucose-1-P, 1% glycogen, and anions at the indicated concentrations. At 5 min, 30 µL were removed, and the radioactivity incorporated into glycogen was measured as described under Materials and Methods. (B) Conditions were described in (A) except that no AMP was used and pyridoxal-reconstituted phosphorylase *b* was substituted by pyridoxal-reconstituted phosphorylase *a* (139 µg/mL)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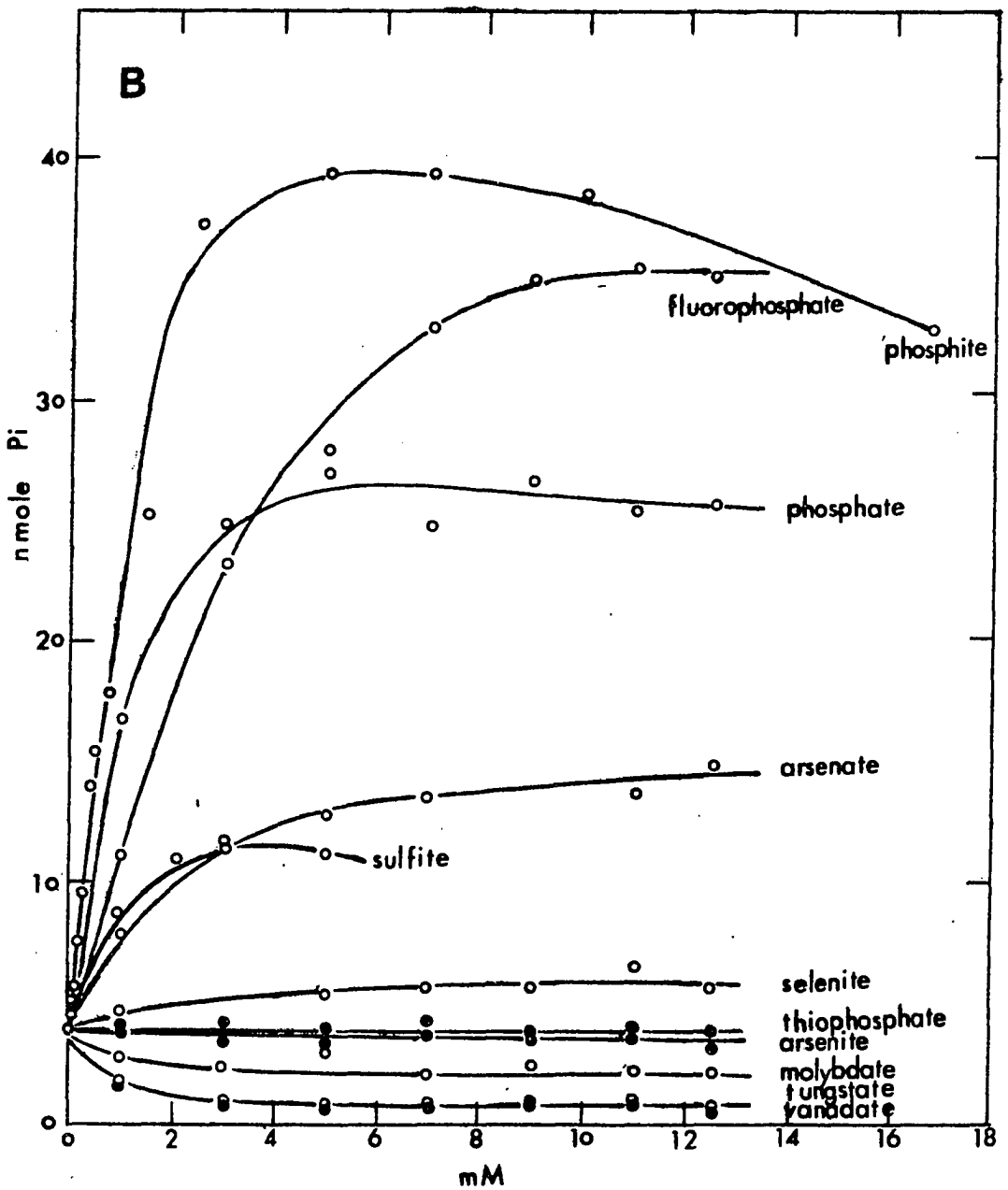


Figure 1 (Continued)

tion cannot be explained by any residual native enzyme in the pyridoxal enzyme preparations. In fact, sulfite, selenite, phosphate, and phosphite but not fluorophosphate at 5 mM caused some inhibition of native phosphorylase b (not illustrated). The extent of activation seen in Figure 1 is likely due to a balance of effects: (a) activation caused by binding at the coenzyme phosphoryl binding site and (b) inhibition by binding at the glucose-1-P site (4). The apparent kinetic constants of the activators used in Figure 1 along with thiophosphate and some alkylphosphonates for pyridoxal-reconstituted phosphorylase b are shown in Table 1.

In Table 1, a great variation of K_m's (from 0.4 to 28.6 mM) but a comparably smaller variation of V_{max}'s (from 0.5 to 4.7 IU) is seen among the different phosphate analogues. The distribution of K_m values seems to be dependent on the pK_{a2} values. Analogues with pK_{a2} higher than 7.1 have considerably higher K_m values. One exception of this generality is (aminomethyl) phosphonate, which has a low pK_{a2}, 5.9, and a high K_m value. The observed dependence of K_m value on pK_{a2} can be explained reasonably by assuming that the binding between phosphate analogues and protein is mainly through an electrostatic interaction; therefore, the analogues with higher pK_{a2}'s interact more weakly with protein because they carry less negative charge. Although (aminomethyl)phos-

Table 1. Apparent kinetic parameters of activator anions on the pyridoxal-reconstituted phosphorylase b[a]

Anion	pK_{a2}	V_{max} [μmol/(min·mg)]	K_M (mM)
phosphite	6.6[b]	4.7	0.6
fluorophsphate	4.8[c]	3.9	1.1
phosphate	7.7[c]	3.6	1.3
thiophosphate	6.2[d]	1.8	0.4
(aminomethyl)phosphonate	5.9[d]	2.0	12.1
ethylphosphonate	8.4[d]	2.6	28.6
methylphosphonate	7.9[d]	2.6	24.0
selenite	8.3[e]	1.8	3.1
sulfite	7.0[f]	1.1	1.1[h]
thiosulfate	2.3[g]		0.8
arsenate	6.9	0.5	1.9

[a]The reaction mixture at 30°C and pH 6.8 contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.016 M glucose-1-P, 1% glycogen, varied concentrations of different anions (except for thiosulfate), and pyridoxal-reconstituted phoshporylase b (∿200 μg/mL).

[b]Weast (26).

[c]Van Wazer (27).

[d]Byers et al. (28).

[e]Kudryavtsev (29).

[f]Nickless (30).

[g]Yui and Hugisawa (31).

[h]This is the K_I value evaluated from the secondary plot of Figure 3B.

phonate has a low pK_{a2} value, the partial positive charge carried by the amino group may lower the net negative charge of the whole molecule and weaken its binding to a positively charged site on the protein. Crystallographic studies of phosphorylase have shown that the 5'-phosphoryl group of PLP is surrounded by several basic amino acids. This also indicates that strong electrostatic interactions occur between this phosphoryl group and its surroundings in the protein.

Because the 5'-phosphoryl group of PLP resides closely to the glucose-1-P binding site on the enzyme, a possible function of this phosphoryl group is to provide a certain structural flexibility to the enzyme necessary for the binding of glucose-1-P. To test this possibility, kinetic studies were done to evaluate the binding of phosphite and glucose-1-P with pyridoxal-reconstituted phosphorylase b. Enzymatic activities were measured in the presence of variable phosphite

Scheme I

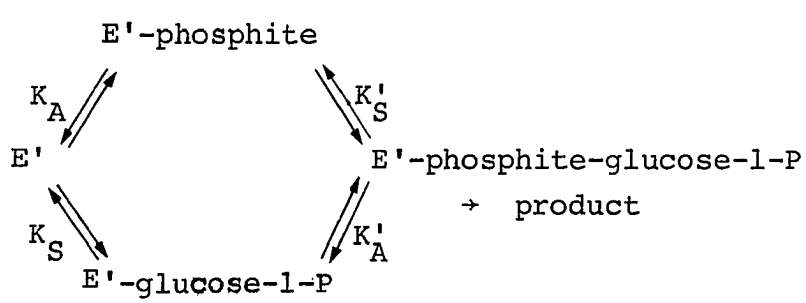
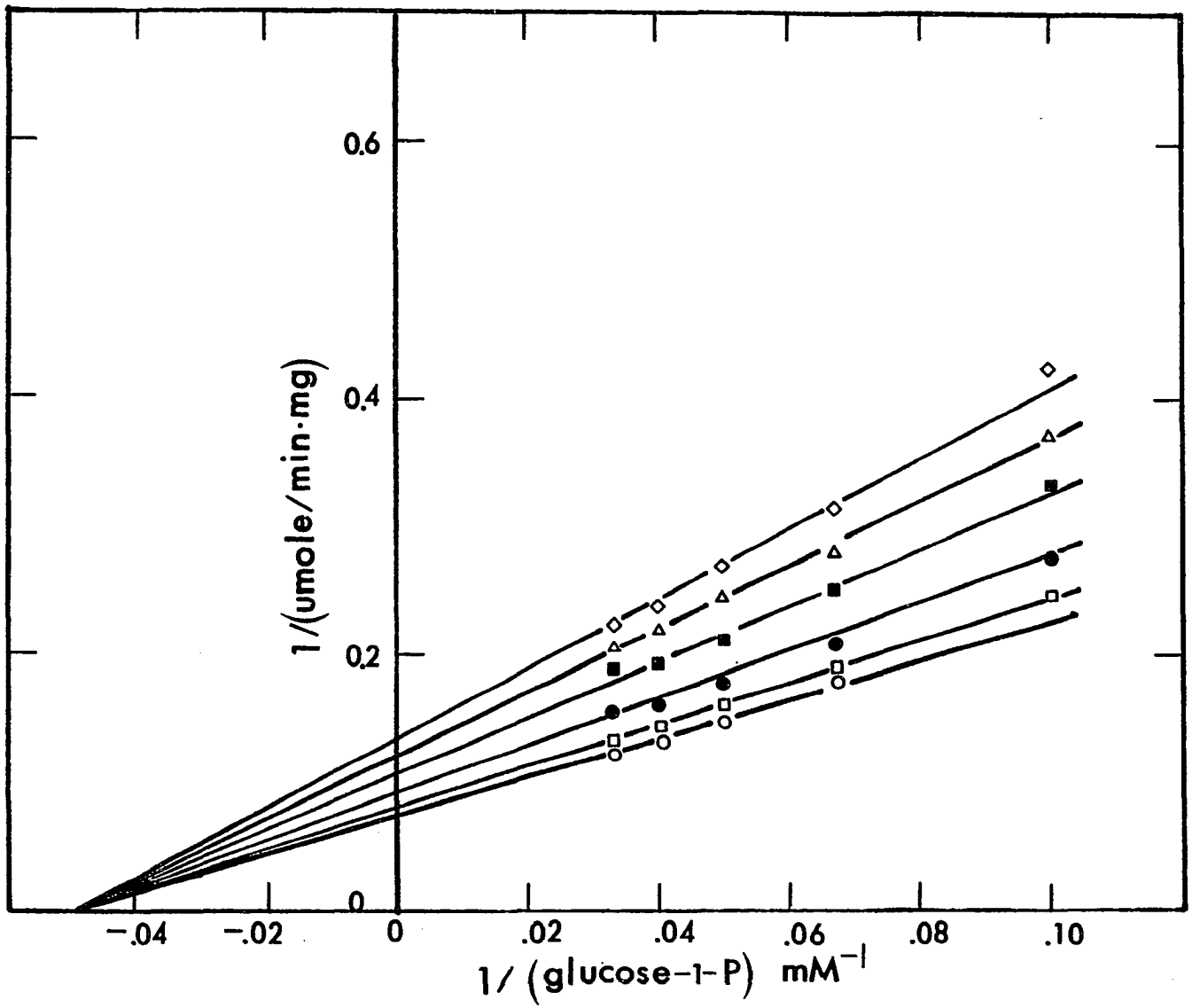


Figure 2. Double-reciprocal plot of initial reaction velocity v vs. [glucose-1-P]. Phosphite concentrations were held constant at 4.0 (O), 3.0 (□), 2.0 (●), 1.0 (■), 0.75 (△), and 0.5 mM (◇). The reaction mixtures at pH 6.8 and 30°C consisted of pyridoxal-reconstituted phosphorylase b (155 μg/mL), 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.001 M AMP, nad 1% glycogen with glucose-1-P and phosphite at the indicated concentrations


0.6
0.4
0.2
0
1/(umole/min·mg)
−.04
−.02
0
.02
.04
.06
.08
.10
1/ (glucose-1-P) mM⁻¹

and glucose-1-P concentrations, and these data are shown in the Lineweaver-Burk plot of Figure 2. The arrangement of these lines in Figure 2, converging at the x axis, can be described by an equation derived for either a random or an ordered bireactant mechanism. Withers et al. (32) found a parallel line pattern for a similar kinetic study in 100 mM KCl and suggested a sequential mechanism. Because we found the K_m value for phosphite is raised by 100 mM KCl from 0.6 to 1.5 mM (results not illustrated), salt might allow binding of phosphite at both inhibitor and activator sites and thus provide an explanation for the differences in our experiment from that of Withers et al. (32). Because phosphite has dual effects, inhibition and activation, on pyridoxal-reconstituted phosphorylase, the kinetic data obtained (Figure 2) might be more complicated than a simple sequential mechanism. To simplify the interpretation of the kinetics, we also used fluorophosphate, another activator anion for the pyridoxal enzyme that showed no inhibition even up to 25 mM (Figure 1), to study the activation process. The resulting double-reciprocal plot showed similar patterns to that of Figure 2, i.e., a set of linear lines converging at the x axis. This observation suggests that a sequential mechanism exists. Fromm's kinetic approach (33) was used to differentiate between random and ordered mechanisms. Scheme 1 represents a random mechanism for the addition of glucose-1-P

and an activator, phosphite.

The rate equation for this mechanism in the presence of a competitive inhibitor for phosphite (I_b) is

$$\frac{V_{max}}{v} = 1 + \frac{K_{S'}}{[S]} + \frac{K_{A'}}{[A]} + \frac{K_A K_{S'}}{[A][S]} + \frac{K_A K_{S'}[I_b]}{K_{I_b'}[A][S]} + \frac{K_{A'}[I_b]}{K_{I_b}[A]} \quad (1)$$

where E', A, S, $K_{I_b'}$, are respectively enzyme complex saturated with glycogen and AMP, phosphite, glucose-1-P, dissociation constant of the inhibitor from I_b-E' complex, and dissociation constant of inhibitor from I_b-E'-glucose-1-P complex. Competitive kinetics with respect to phosphite and noncompetitive kinetics with respect to glucose-1-P are predicted from Equation 1. On the basis of the same scheme, the equation in the presence of a competitive inhibitor (I_a) for glucose-1-P can be described as

$$\frac{V_{max}}{v} = \frac{K_{S'}}{[S]} + \frac{K_{A'}}{[A]} + \frac{K_A K_{S'}}{[A][S]} + 1 + \frac{K_A K_{S'}[I_a]}{K_{I_a'}[A][S]} + \frac{K_{S'}[I_a]}{K_{I_a}[S]} \quad (2)$$

where K_{I_a} and $K_{I_a'}$, are the dissociation constants for the interaction of the inhibitor with E' and E'-A complex, respectively. Noncompetitive kinetics with respect to phosphite and competitive kinetics with respect to glucose-1-P are predicted from Equation 2. With ordered mechanisms, a competitive inhibitor for the second substrate will yield uncompetitive kinetics with respect to the first substrate.

Figure 3A. Double-reciprocal plots of thiosulfate inhibition of pyridoxal-reconstituted phosphorylase. The reaction mixtures at pH 6.8 and 30°C consisted of pyridoxal-reconstituted phosphorylase (192 µg/mL), 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.001 M AMP, and 1% glycogen. Glucose-1-P at 0.016 M, phosphite at the indicated concentrations and thiosulfate at 0 (O), 1.0 (□), 2.0 (△), and 4 mM (◐)

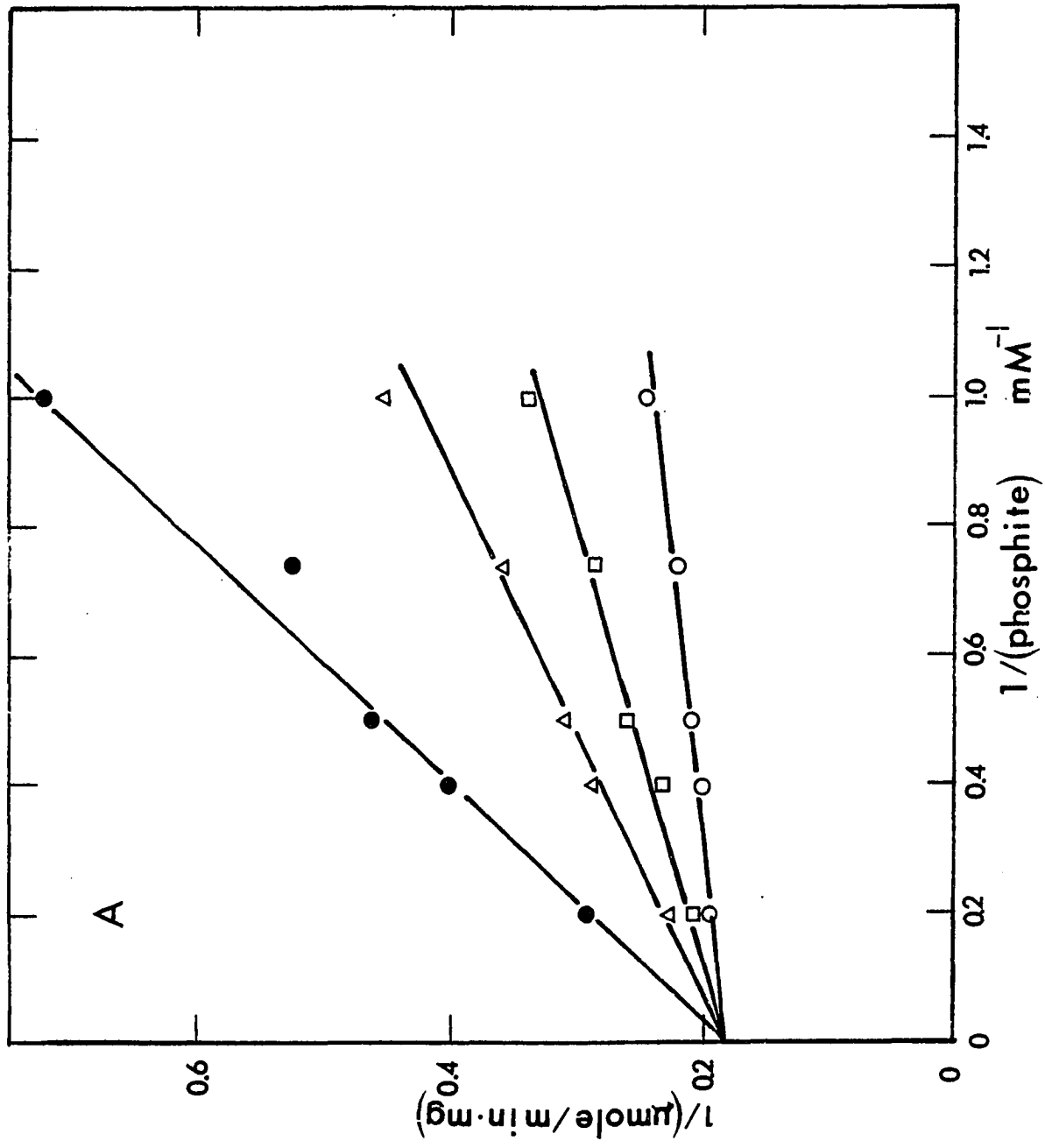
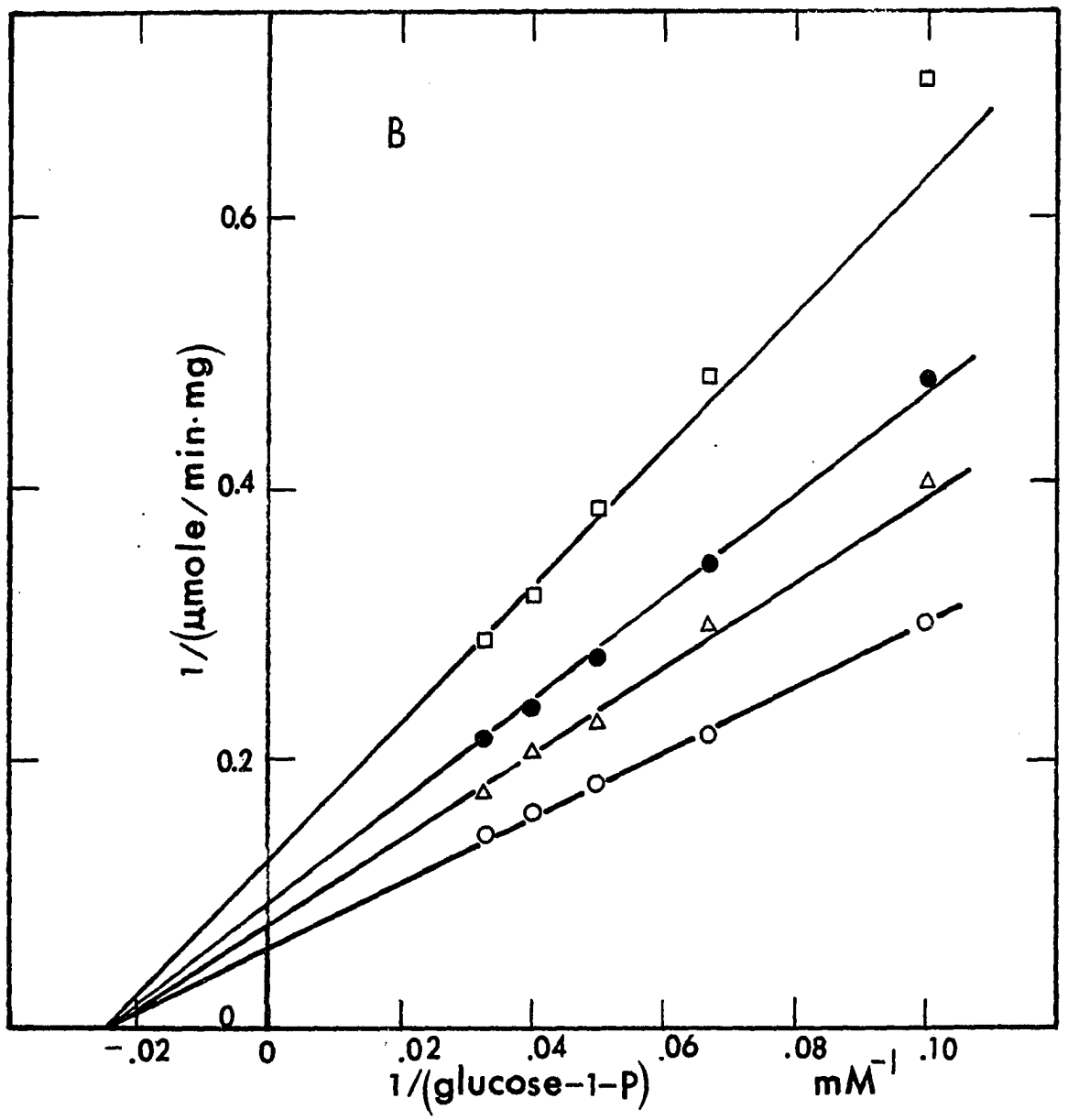

A
1/(μmole/min·mg)
0
0.2
0.4
0.6
1/(phosphite) mM⁻¹
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4

Figure 3B. Double-reciprocal plots of thiosulfate inhibition of pyridoxal-reconstituted phosphorylase. The reaction mixtures at pH 6.8 and 30°C consisted of pyridoxal-reconstituted phosphorylase (192 µg/mmL), 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.001 M AMP, and 1% glycogen. Phosphite at 0.002 M, glucose-1-P at the indicated concentrations, and thiosulfate at 0 (O), 1.0 (Δ), 2.0 (●), and 4 mM (□)


B
0.6
0.4
0.2
0
1/(μmole/min·mg)
-.02
0
.02
.04
.06
.08
.10
1/(glucose-1-P)
mM⁻¹

The unique inhibitory pattern permits a distinction to be made between ordered and random bireactant kinetic mechanisms.

Thiosulfate is a competitive inhibitor for phosphite (Figure 3A), but it is a noncompetitive inhibitor with respect to glucose-1-P (Figure 3B). That thiosulfate (up to 20 mM, data not shown) did not affect the enzymatic activity of the native phosphorylase b also indicates that this anion cannot compete with glucose-1-P for the same binding site in the protein. Glucose cyclic 1,2-phosphate is a good competitive inhibitor for glucose-1-P in native phosphorylase (34). This cyclic compound is a good competitive inhibitor for glucose-1-P in pyridoxal-reconstituted phosphorylase b and a noncompetitive inhibitor for phosphite as demonstrated by Withers et al. (32). Because no uncompetitive kinetic pattern was observed and the results fit the pattern predicted by Equations 1 and 2 derived from Scheme 1, the studies support strongly a rapid equilibrium random Bi-Bi mechanism for pyridoxal phosphorylase. The constants, K_A, $K_{A'}$, K_S, and $K_{S'}$, were evaluated from Figure 2 and the same kinetic study with fluorophosphate instead of phosphite, by using Equations 1 and 2, and are shown in Table 2. That the dissociation constants K_S and K_A are virtually equal to $K_{S'}$ and $K_{A'}$, respectively, shows the binding of activator, either fluorophosphate or phosphite, and glucose-1-P are independent events. If the pyridoxal enzyme-phosphite

Table 2. Kinetic parameters of pyridoxal-reconstituted phosphorylase b activated by phosphite and fluorophosphate[a]

	Phosphite	Fluorophosphate
V_{max} [μmol/(min·mg)]	15.4	7.7
K_A (mM)	0.8	1.6
$K_{A'}$ (mM)	0.6	1.5
K_S (mM)	18.0	22.3
$K_{S'}$ (mM)	22.0	23.8

[a]Calculations are described under Results and Discussion.

complex is a good model for coenzyme binding in native phosphorylase, the results also imply that the 5'-phosphoryl group of PLP in native phosphorylase is not needed for glucose-1-P binding, although these two sites are close by in protein. The pyridoxal enzyme-phosphite complex showed weaker affinity for glucose-1-P ($K_{S'}$ = 22 mM) and lower V_{max} (15.4 IU) than those of native phosphorylase [K_m(glucose-1-P) = 7.4 mM and V_{max} = 65 IU (35). This observation indicates that the linkage between the phosphoryl group and pyridoxal influence glucose-1-P binding and catalytic capability of phosphorylase. Pfeuffer et al. (36) showed that PLP molecule is important for the integrity of the native phosphorylase. In the pyridoxal enzyme-phosphite complex, a weak H bond between HPO_3^{2-} and 5-CH_2OH of pyridoxal may partly replace

Figure 4A. Double-reciprocal plots of molybdate inhibition of pyridoxal-reconstituted phosphorylase b. Reaction mixtures at pH 6.8 and 30°C consisted of 0.04 M β-glycerophosphate, 0.002 M EDTA, 0.001 M AMP, 1% glycogen, and 225 μg/mL pyridoxal-reconstituted phosphorylase b. [U-^{14}C]Glucose-1-P at 0.016 M, phosphite at the indicated concentrations, and molybdate at 0 (□), 1.25 (■), 3.0 (O), and 5 mM (●)

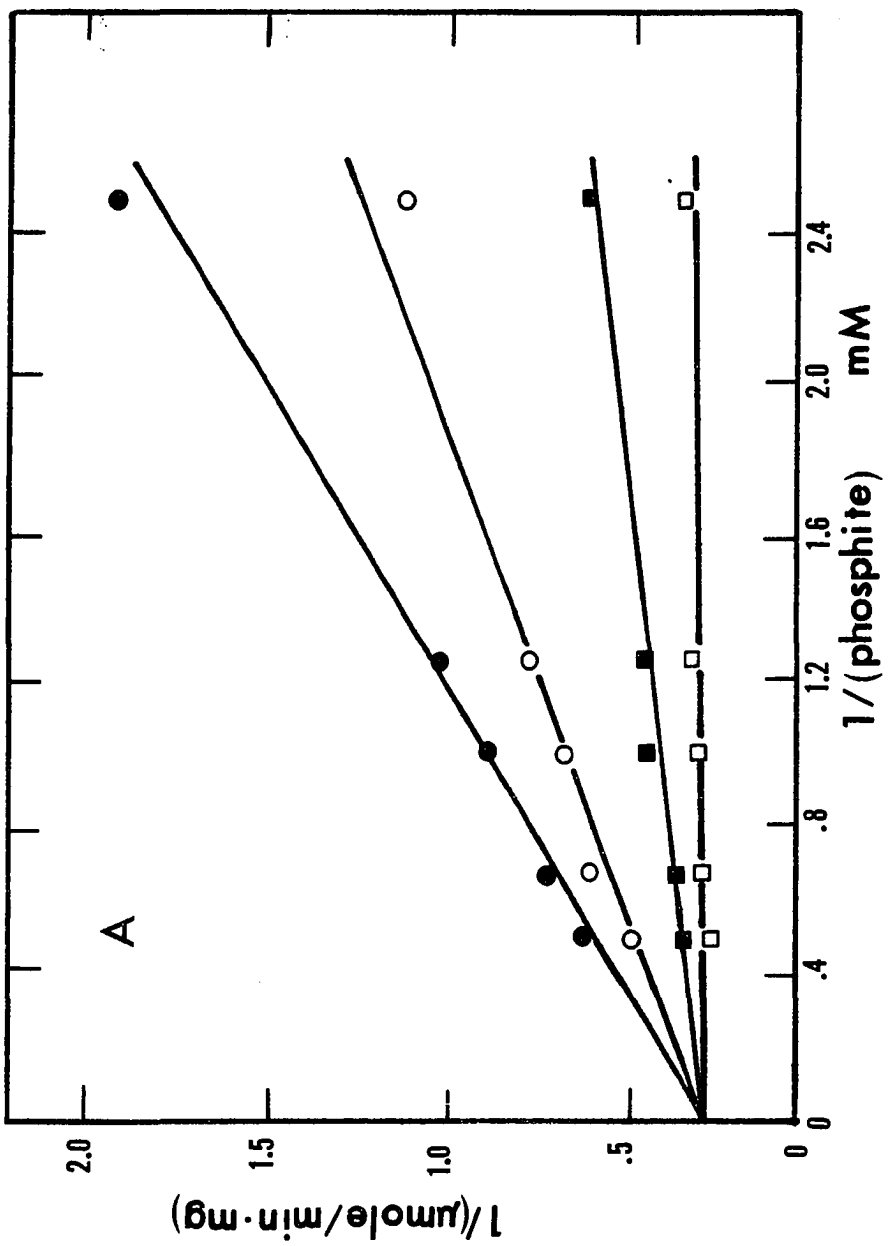
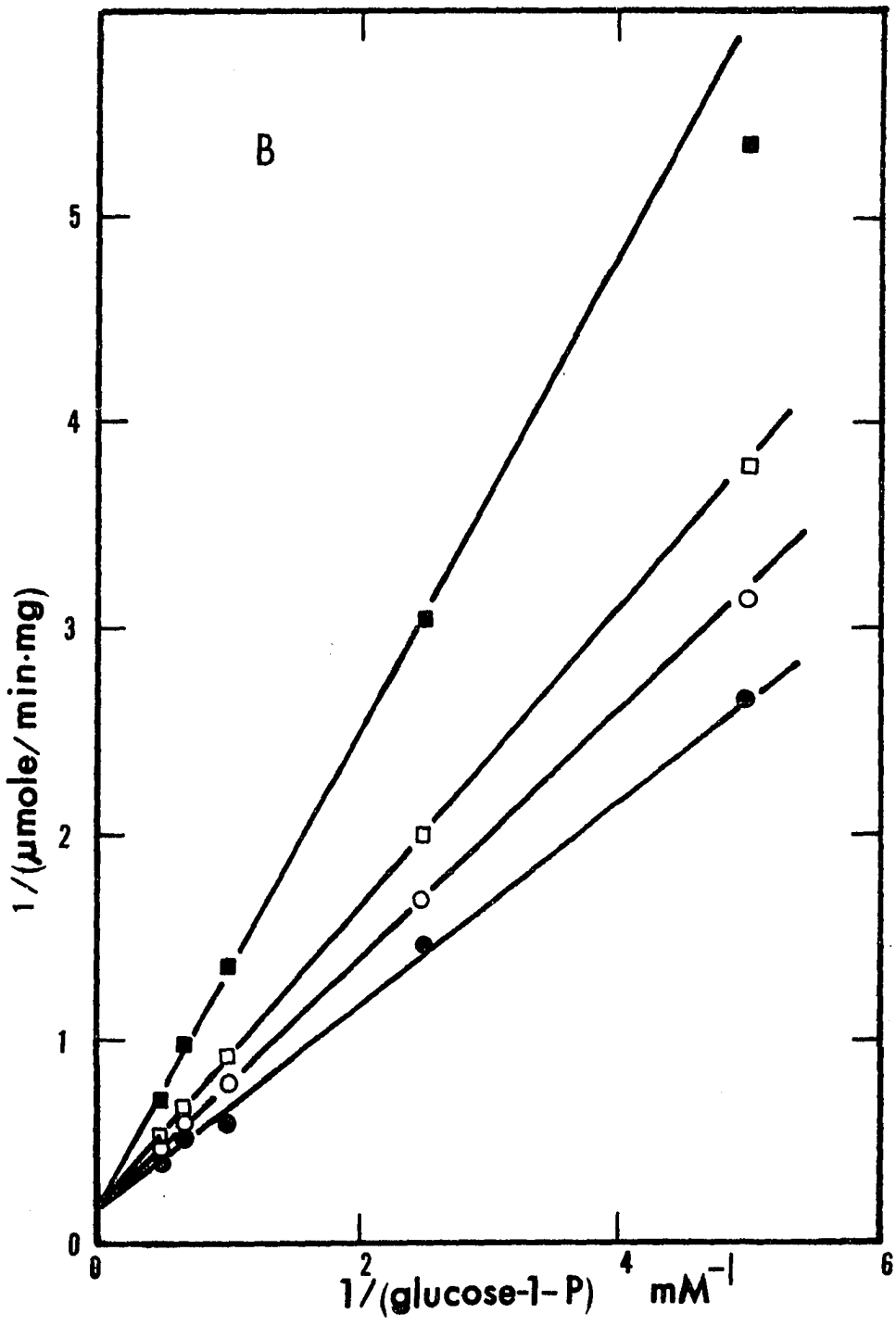

A
1/(μmole/min·mg)
2.0
1.5
1.0
.5
0
0
.4
.8
1.2
1.6
2.0
2.4
1/(phosphite) mM

Figure 4B. Double-reciprocal plots of molybdate inhibition of pyridoxal-reconstituted phosphorylase b. Reaction mixtures at pH 6.8 and 30°C consisted of 0.04 M β-glycerophosphate, 0.002 M EDTA, and 0.001 M AMP, 1% glycogen, and 225 μg/mL pyridoxal-reconstituted phosphorylase b. Phosphite at 0.002 M, [U-^{14}C]glucose-1-P at the indicated concentrations, and molybdate at 0 (●), 1.0 (○), 2.0 (□), and 5.0 mM (■)


B
5
4
3
2
1
0
0
2
4
6
1/(µmole/min·mg)
1/(glucose-1-P) mM⁻¹

the missing covalent bonding and lead to a recovery of up to 30% enzymatic activity. In the 5'-deoxypyridoxal-reconstituted phosphorylase, the small activity recovered by phosphite (∿0.7% of the native phosphorylase) might be explained by the lack of hydrogen-bond potential between HPO_3^{2-} and the 5-CH_3 on the 5'-deoxypyridoxal molecule. Because the linkage between the phosphoryl group and pyridoxal is important for catalysis and the phosphoryl group of PLP binds strongly to the protein, it is possible that one of the functions of the 5'-phosphoryl group of PLP in phosphorylase is to act as an anchoring point and keep the PLP molecule and/or various amino acid residues in proper position for effective catalysis.

Because the phosphoryl group of PLP was suggested to go through a trigonal-bipyramidal intermediate during phosphorylase catalysis (13, 14), we used molybdate, which is monomeric (37) and is believed to be able to mimic the trigonal-bipyramidal state (38). in kinetic studies with pyridoxal phosphorylase to further test this mechanism. Molybdate inhibits the pyridoxal enzyme, and kinetic studies show that it is competitive with respect to both phosphite (Figure 4A) and glucose-1-P (Figure 4B). The apparent K_I's of molybdate toward phosphite and glucose-1-P evaluated from secondary plots of Figure 4 are 0.1 and 2.5 mM, respectively. Because molybdate binds more tightly to the

PLP phosphate site on the enzyme than phosphite (K_m = 0.6 mM), this result suggests that this oxyanion may resemble the transition state of the enzyme-bound phosphite during the catalysis. This transition state that molybdate imitates may be a trigonal-bipyramidal species as suggested by Van Eteen et al. (38). Vanadate and tungstate also can mimic the trigonal-bipyramidal state (38) and are inhibitors of pyridoxal phosphorylase. The exact interpretation of the results is more complex because these oxyanions can exist in different oligomeric states. An account of their effects on phosphorylase is found in the accompanying paper (39). The possibility that the enzyme-bound phosphate is transformed into a trigonal-bipyramidal intermediate during the catalysis is also supported by the observation of the varied effects of phosphate analogues with pyridoxal phosphorylase. When one oxygen atom on the phosphate is substituted by an electron-donating group such as sulfur, methyl, or ethyl, these anions show lower V_{max}'s than phosphate (see Table 1). The low electrophilicity of the center phosphorus atom in these analogues makes them harder to form the trigonal-bipyramidal intermediate needed for the catalysis and might be responsible for their low V_{max} values. On the basis of the differences of effects (K_m and V_{max}) between phosphate analogues and sulfate analogues, the nature of the center atom seems to be important for their effects on pyridoxal enzyme. Thiophosphate is an

activator, but thiosulfate can bind to the protein and inhibit the restoration of enzymatic activity by phosphite. Phosphate can activate, but sulfate (at 1-5 mM), because it does not activate or inhibit, probably does not even bind to the pyridoxal enzyme. These differences can be explained by the fact that phosphorus can more easily expand its valence shell to form a trigonal-bipyramidal structure than sulfur.

Although the results with molybdate and varied phosphate analogues are consistent with a mechanism that a trigonal-bipyramidal intermediate is involved in the catalysis, results in this study are different from those reported previously in other systems. When molybdate was used as a transition-state analogue in phosphatases, it binds to the enzyme 10^3-10^4 times more tightly than phosphate, while the binding of molybdate to pyridoxal enzyme is only 13 times tighter than phosphate. The variation in V_{max}'s among phosphate and its analogues, thiophosphate and methyl- or ethylphosphonate, in this study (see Table 1) is much smaller than those found in hydrolytic reactions among phosphoester and its analogues (40), which were believed to proceed through a trigonal-bipyramidal intermediate. These differences could be explained by either that the structural transformation of PLP phosphate into a trigonal-bipyramidal species is not the rate-limiting step during the catalysis or that the hypothesis that PLP phosphate forms

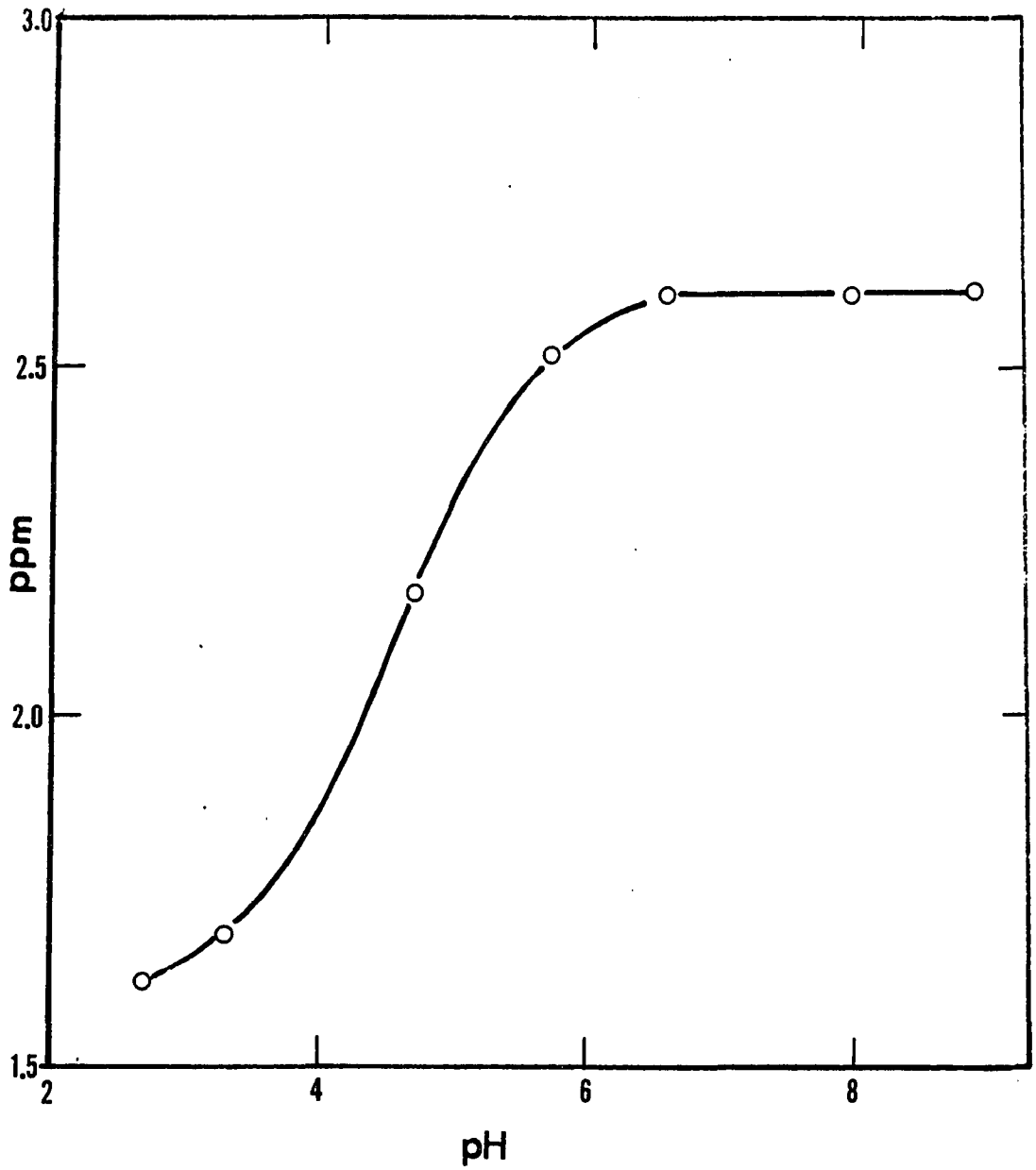
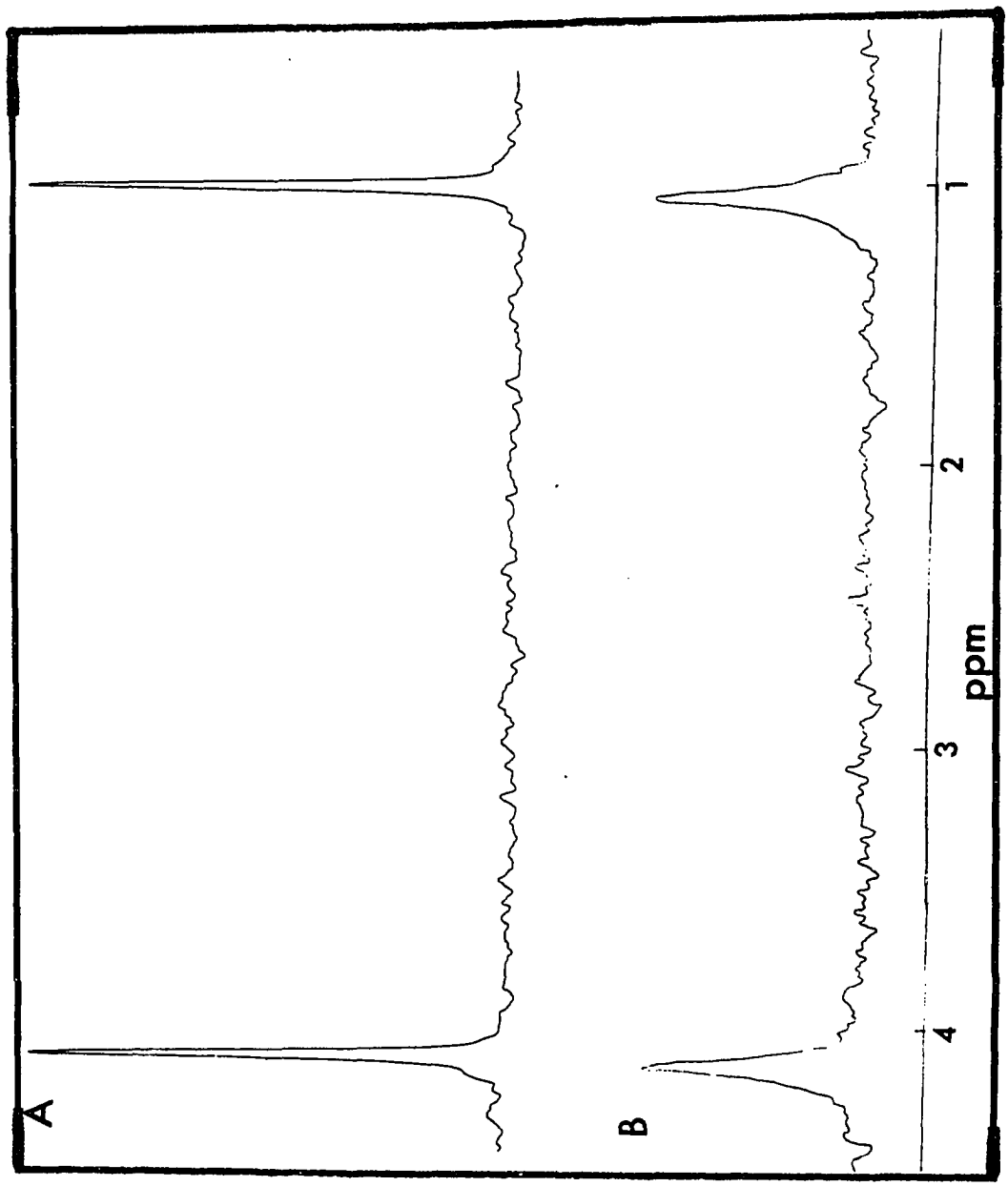


Figure 5. ^{19}F NMR chemical shift, average of the doublet in each ^{19}F NMR spectrum, of fluorophosphate at different pH values. Conditions were as described under Materials and Methods

a trigonal-bipyrimidal intermediate is not a perfect description of the real mechanism of phosphorylase. Therefore, other mechanisms with the involvement of PLP in the catalysis of phosphorylase cannot be excluded.

Because fluorophosphate is a good activator of pyridoxal phosphorylase and has a low pK_a value (4.8), but the pyridoxal enzyme activated by fluorophosphate does not have a different pH profile than that of phosphate, Withers et al. (41) suggested that it is unlikely that the phosphoryl group of PLP in phosphorylase is involved in a proton shuttle. However, if a change in the pK_a value of fluorophosphate occurred upon binding, a similar pH profile might be obtained for the enzyme activated by the two anions - even if the phosphoryl group was involved in a proton-shuttle mechanism. ^{19}F NMR spectroscopy was used to examine different ionization states of fluorophosphate. Because the fluorine nucleus is coupled by the adjacent phosphorus (I = 1/2), the ^{19}F NMR spectrum of fluorophosphate shows a doublet. ^{19}F NMR spectra of fluorophosphate have been followed at varied pH's, and the averages of the chemical shifts of the doublets are shown in Figure 5. A transition of the ^{19}F resonance in Figure 5 is found between pH 2 and 6, and the midpoint of this transition is measured to be 4.5, which corresponds to the pK_a of fluorophosphate determined by other methods (27). ^{19}F NMR spectra

Figure 6. Fluorine magnetic resonance spectrum (A) Fluorine magnetic resonance spectrum of 0.00023 M fluorophosphate in a solution at pH 6.8 consisting of 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.001 M AMP, and 0.002 M EDTA, 18064 acquisitions. (B) Fluorine nuclear magnetic resonance spectrum of a similar solution as in (A) with added pyridoxal-reconstituted phosphorylase b (21.7 mg/mL), 18064 acquisitions. Conditions were as described under Materials and Methods


A
B
4
3
2
1
ppm

of 0.23 mM fluorophosphate in the absence and presence of an equal concentration of pyridoxal phosphorylase b have been obtained and are shown in Figure 6. The broadening of ^{19}F NMR signals of free fluorophosphate upon addition of the enzyme indicates that part of fluorophosphate is bound to the protein. The two sets of resonances in Figure 6 have identical integration of their areas relative to the external referencing signal (TFA). The fluorophosphate signals shifted slightly downfield ($\sim$0.04 ppm) in the presence of enzyme. This difference might be explained by the differences in susceptibility of the external standard or some mechanism other than a protonation process, which would shift the signals upfield. This observation shows that the binding to the pyridoxal enzyme does not change the ionization state of fluorophosphate. Phosphate (58 mM), another activator, which was added to the fluorophosphate and enzyme mixture to release the bound fluorophosphate from the enzyme, caused the ^{19}F signals to become the same width as the signal obtained from free fluorophosphate, while the area under the ^{19}F signals remained unchanged. Mixing fluorophosphate (1 mM) with enzyme (0.2 mM), AMP (1 mM), glucose-1-P (15 mM), and maltopentaose (1%) causes the half-height width of the ^{19}F signals to decrease from 19.9 Hz (measured at 8 min after mixing) to a steady value of 13 Hz (after 20 min), while the chemical shift

and the areas of ^{19}F resonance measured at different times remained unchanged. The gradual narrowing of ^{19}F signals as the reaction proceeded indicates that the increasing amount of phosphate produced can release fluorophosphate from the protein by competing for the same binding site on protein. Because no detectable change of the fluorophosphate signal positions occurred during catalysis, the results indicate that no appreciable change in the ionization state of bound fluorophosphate happens during the reaction. However, it is possible that a proton shuttle might not be detected by NMR spectroscopy if the steady-state concentration of a protonated enzyme form, which may exist transiently during the catalysis, is low.

In summary, the data in this study are consistent with a mechanism in which the 5'-phosphoryl group of PLP forms a trigonal-bipyramidal intermediate during the catalysis. Phosphate analogues bound to the pyridoxal phosphorylase, and possibly the 5'-phosphoryl group of PLP in native enzyme, are unlikely to undergo a protonation-deprotonation process. Kinetic study indicates that glucose-1-P does not need the phosphoryl group of PLP for its binding to phosphorylase. The dianionic state of the PLP phosphate is crucial for this coenzyme to perform its functions, one of which could be holding a proper active site region for effective catalysis. Other

functions of PLP in phosphorylase are still possible and need to be further explored.

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SECTION 2: CHEMICAL SYNTHESIS AND PROPERTIES OF 6-FLUORO-PYRIDOXAL PHOSPHATE. STUDIES OF GLYCOGEN PHOSPHORYLASE RECONSTITUTED WITH 6-FLUOROPYRI-DOXAL AND 6-FLUOROPYRIDOXAL PHOSPHATE

ABSTRACT

6-Fluoropyridoxal phosphate has been synthesized. Its properties were studied, and it was used, along with 6-fluoropyridoxal, to reconstitute apophosphorylase b. Kinetic studies of the resulting enzymes showed that phosphorylases reconstituted with 6-FPLP and 6-FPAL have characteristics similar to those of native and pyridoxal enzyme, respectively, except that the former two enzymes have lower V_{max} values. ^{19}F NMR and UV spectra of 6-FPLP phosphorylase showed that the coenzyme forms a neutral enol-imine Schiff's base. Because the UV and fluorescence spectra of 6-FPLP phosphorylase are comparable to those obtained with native phosphorylase, it further confirms the postulate that PLP forms a neutral enolimine Schiff's base in phosphorylase. ^{19}F NMR study of 6-FPLP and 6-FPAL reconstituted phosphorylase in the inactive and active state indicates that the 3-phenolic group of the coenzyme is unlikely to be involved in an acid-base reaction essential for the catalysis of phosphorylase. However, the comparison of the properties of 6-FPLP reconstituted and native phosphorylases implies that the ring nitrogen of the coenzyme PLP in phosphorylase may interact with the protein during catalysis, and this interaction is important for the efficient catalysis of phosphorylase.

INTRODUCTION

Glycogen phosphorylase contains stoichiometric amount of pyridoxal 5'-phosphate (1). Although the exact details of the function of this coenzyme in phosphorylase have not been elucidated, various studies showed that the coenzyme is an indispensable constituent for both the structural integrity and the enzymatic activity of phosphorylase. That PLP is a structural determinant of phosphorylase has been demonstrated by the facts that apophosphorylase a and b are less stable than the native phosphorylase (2,3) and that the quaternary structures of apoproteins are quite different from those of native enzymes. Although the direct involvement of PLP in the catalysis of phosphorylase has not been clearly demonstrated yet, the studies of phosphorylase reconstituted with various PLP analogues suggested that the 5'-phosphoryl group of enzyme-bound PLP is likely to be involved in catalysis because the phosphorylase reconstituted with most of analogues of PLP modified at the 5'-position were inactive (4,5). Besides, X-ray crystallographic studies of phosphorylase a and b show that the phosphoryl group of the substrate, glucose-1-P, when bound to the active site is about 7 Å from the phorphoryl group of the coenzyme (6,7). Because the phosphoryl group of coenzyme is absolutely required for enzymatic activity and this phosphate resides next to the

enzyme-bound substrate, this particular group has been suggested to act as electrophile (8,9), nucleophile (10), or proton-donor (7,11) during catalysis. Phosphorylases reconstituted with PLP modified at the 3-position, O-methyl pyridoxal phosphate (12), and at the 1-position, N-methyl pyridoxal phosphate (13) and pyridoxal phosphate N-oxide (14), have also been studied. Phosphorylase reconstituted with O-methyl pyridoxal phosphate was active, suggesting that the dissociable hydrogen of the phenolic group is not involved in a proton shuttle necessary for enzymatic activity. However, the significant steric effect due to the presence of a methyl group in the coenzyme makes the interpretation of this result rather complicated. Because N-methyl pyridoxal phosphate does not bind to apoenzyme and pyridoxal phosphate N-oxide binds to the apoenzyme but is gradually converted to PLP when it is bound to the enzyme, the function of the ring nitrogen of PLP in phosphorylase is still unclear.

In order to study the importance of these functional groups and possible change around the coenzyme binding site in the active phosphorylase, we have synthesized 6-fluoropyridoxal phosphate and studied the properties of phosphorylase reconstituted with it. The substitution of 6-hydrogen of PLP by a fluorine atom does not cause severe steric effects because these two atoms have similar diameters.

However, because fluorine is very electronegative, its inductive effect can affect the apparent pK_a values of the ring nitrogen and the 3-phenolic groups of 6-FPLP in aqueous solution as reported by Korytnyk and Kravastava (15) with 6-fluoropyridoxal. Therefore, fluorinated PLP analogues are ideal for the task of studying whether these groups are involved in any acid-base interaction which is essential for phosphorylase catalysis. Besides, because ^{19}F is a sensitive NMR nucleus, this nucleus on the enzyme-bound 6-FPLP can serve as a reporting group, monitored by ^{19}F NMR spectrometer, to provide us with more insights about the coenzyme and its environment inside phosphorylase. A preliminary account of this work has been reported (16).

MATERIALS AND METHODS

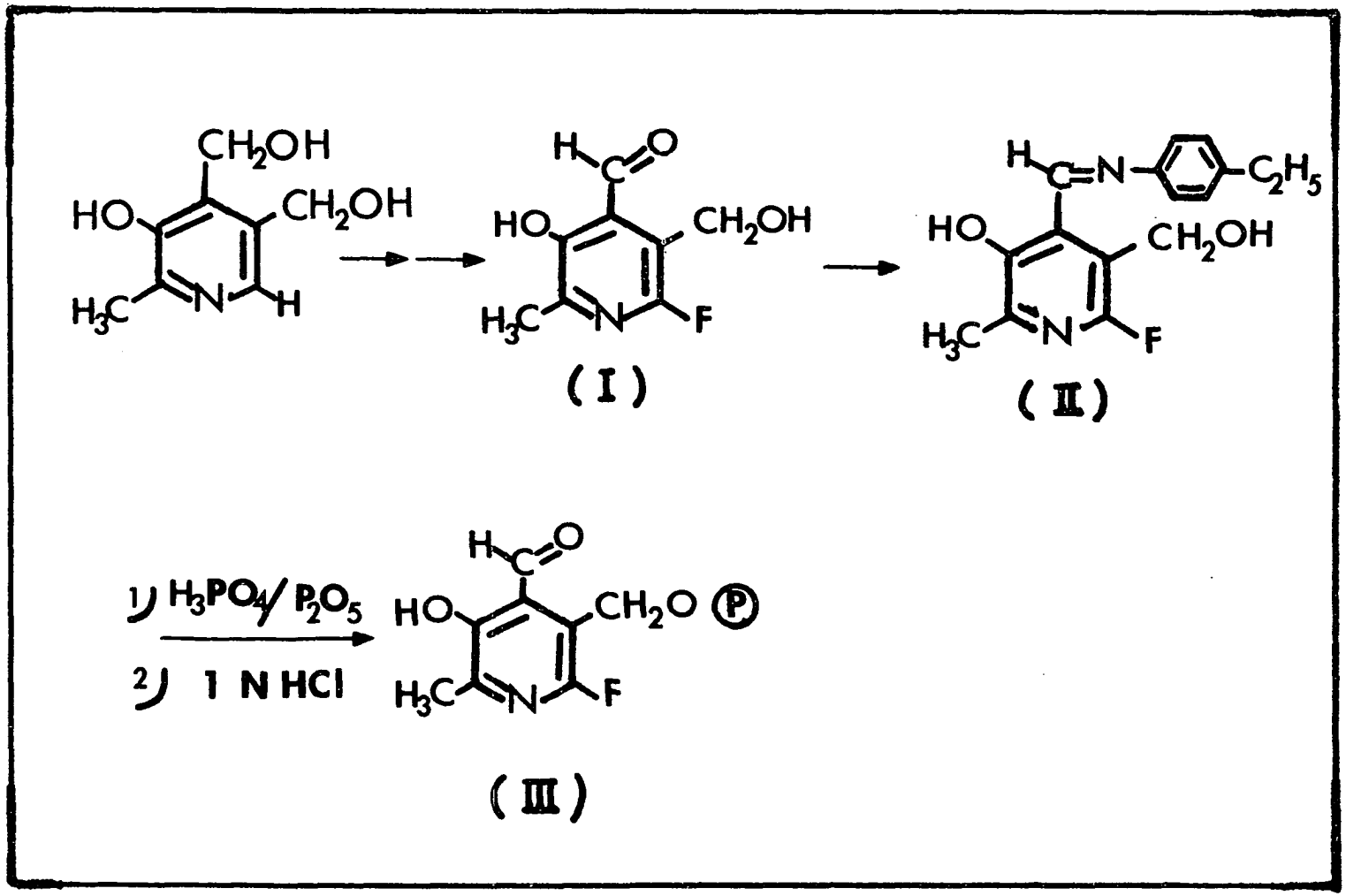
Rabbit skeletal muscle was obtained from Pel-Freeze Co. The purchase of [U-^{14}C] glucose-1-P was made from Amersham. Pyridoxine hydrochloride was purchased from National Biochemical Corporation. Pyridoxal, pyridoxal 5'-phosphate, and glucose-1-P were obtained from Sigma Company. P-phenetidine was obtained from Alfa Products.

Chemical Synthesis of 6-FPLP and 6-FPAL

The method that we used to synthesize 6-FPLP starts with 6-FPAL (I, Figure 1). The method described by Korytnyk and Kravastava (15) was used to synthesize 6-FPAL (I). The Schiff's base of 6-FPAL and p-phenetidine (II) was made by adding a solution of 1 ml redistilled p-phenetidine and 13 ml of 1N HCl to a 200 ml aqueous solution containing 0.67 gm of 6-FPAL, and followed immediately by 200 ml of 2 M sodium acetate. The mixture was kept on ice for 3 hours, after which crystals were filtered off, washed by cold water, and dried in a desiccator. The dry weight of the resulting Schiff's base (II) was 0.49 gm.

In a 50-ml round-bottomed flask fitted with a drying tube, 5.2 gm of phosphoric acid (85%) and 4 gm of phosphorus pentoxide were mixed and heated in a boiling waterbath for 30

Figure 1. Chemical synthesis of 6-fluoropyridoxal (I), the Schiff's base of 6-fluoropyridoxal (II), and 6-fluoropyridoxal phosphate (III)


CH2OH
HO
CH2OH
H3C
N
H
H
O
HO
CH2OH
H3C
N
F
(I)
H
N
C2H5
HO
CH2OH
H3C
N
F
(II)
1) H3PO4/P2O5
2) 1 N HCl
H
O
HO
CH2O
P
H3C
N
F
(III)

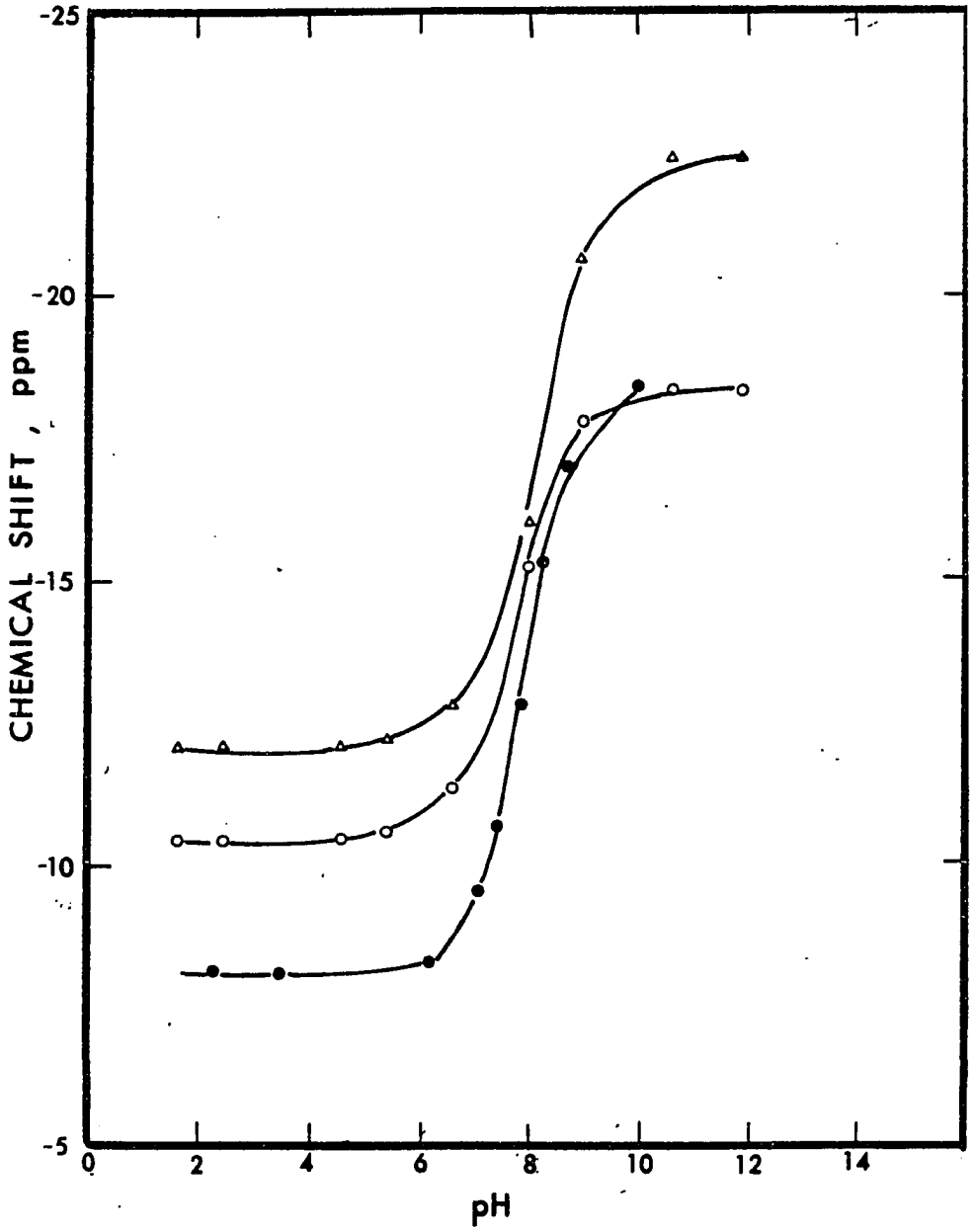
minutes. To the cooled mixture, 0.23 mmole (69 mg) of compound (II) was added. The red, viscous mixture was mixed thoroughly and incubated in a 50°C waterbath for 8 hours under bubbling nitrogen. Then, the reaction mixture was cooled on ice, 2.5 ml of 1 N HCl was added, and the resulting mixture was then heated to 70°C for 10 minutes. The reaction mixture was diluted with 5 ml of degassed water and applied to a 1.25 x 34 cm column of Dowex 50 W x 4 (200-400 mesh) and washed with degassed water. Fractions containing 6-FPLP (III) as identified by their UV-VIS spectra were eluted in the breakthrough fraction, pooled, and kept on ice overnight. Phosphate crystals formed and were filtered off from the solution, and the filtrate was neutralized by adding 10 N NaOH. To the neutralized solution, 4 ml of 0.17 M p-phenetidine was added. The mixture was kept on ice for 6 hours. Crystals (the Schiff's base of 6-FPLP and p-phenetidine) were filtered off, washed extensively with cold water, and dried in a desiccator. The dried crystals were dissolved in 2 ml of 1 N HCl, heated in a boiling waterbath for 1 minute, applied on to a Dowex 50 W x 4 (5-ml) column, and then eluted with degassed water. Fractions with the UV-VIS spectrum of 6-FPLP were pooled and dried by lyophilization. The yield of 6-FPLP (III) was 21 mg (29%). The compound, identified by reaction with Gibbs' reagent, migrated as a single component

on TLC plates with R_f values as 0.84 and 0.63 when developed in n-butanol and saturating 4N HCl or n-butanol and saturating 1 N HCl, respectively. Analytical HPLC (Beckman Ultrasphere, 25 cm x 4.6 mm ID), eluted with 0.5% acetic acid, also showed a single peak at 355 nm.

Properties of 6-FPLP

The UV-VIS spectrum of 6-FPLP showed absorption maxima at 400 nm (ε = 4,000) and 318 nm (ε = 2,300) in a sodium bicarbonate buffer at pH 10. In 0.1 N HCl, it showed absorption maxima at 355 nm (ε = 3,500) and 295 nm (ε = 1,300). Like PLP, ^{1}H and ^{19}F NMR spectra of 6-FPLP revealed that this compound exists in an equilibrating state between an aldehyde and a hydrated aldehyde form. ^{1}H NMR spectrum of 6-FPLP taken at pH 2.0 showed two sets of peaks with a constant ratio of integrations (3.6:1). The peaks of the major form are at 10.5 (s,1H), 5.3 (dt, 2H, J=7.5 Hz), and 2.5 (s,3H) ppm. The peaks of the other form are at 6.6 (s,1H), 5.0 (dt, 2H, J=6.7 Hz), and 2.4 (s,3H) ppm. The peaks of the first set fall at the same positions as those of PLP in the aldehyde form, and the peaks of the latter set fall at the same positions as hydrated PLP except no peak corresponding to 6-H of PLP was seen in either spectrum (17). Two peaks resonating at -12.1 ppm and -10.4 ppm with the same ratio of integration as that between the aldehyde and hydrated 6-FPLP

Figure 2. ^{19}F NMR chemical shift of 6-FPLP and 6-FPAL at different pH values: (O) 6-FPLP in aldehyde state; (Δ) 6-FPLP in hydrated state; (●) 6-fluoropyridoxal. Conditions were as described under Materials and Methods


-25
-20
-15
-10
-5
CHEMICAL SHIFT , ppm
0
2
4
6
8
10
12
14
pH

found in ^{1}H NMR spectrum, 3.6:1, were observed in the ^{19}F NMR spectrum of 6-FPLP taken at pH 2.0 and room temperature. According to the assignments of the same compound in ^{1}H NMR spectrum, resonances at -12.1 and -10.4 ppm can be assigned to aldehyde state and hydrated state of 6-FPLP, respectively. When 6-FPLP was heated to 90°C, only one ^{19}F NMR signal at -11.24 ppm was seen. After the same sample was cooled to room temperature, its ^{19}F NMR spectrum returned to the original pattern. This reversible, temperature dependent change of ^{19}F NMR spectra can be interpreted as due to the existence of a slow equilibrium between aldehyde and hydrated states of 6-FPLP, and an equilibrium that shifts to the aldehyde form at high temperature. A similar observation has been made on pyridoxal in alkaline conditions by Turchin et al. (18).

The pH dependence of ^{19}F NMR chemical shifts of 6-FPAL is shown in Figure 2. The pK_a determined from this curve is 8.0, which is very similar to the pK_a of 3-phenolic OH on the same compound determined by a spectrophotometric method (15). pK_a values of 6-FPLP in the aldehyde and hydrated states, determined from curves in Figure 2, are 8.1 and 7.9, respectively. 6-FPLP showed a pK_a value of 7.8 when analyzed by the spectrophotometric method. Like the assignment made for 6-FPAL, these pK_a's can be assigned to the 3-OH group of

6-FPLP. No other transition is seen from either curve in Figure 2, even at pH 2, and this observation is consistent with Korytnyk and Kravastava's (15) finding that the substitution of 6-H by a fluorine atom can dramatically lower the pK_a of the neighboring N group.

Enzymes

Rabbit skeletal muscle glycogen phosphorylase was prepared according to the method of Fischer and Krebs (19) except that cysteine was substituted by 30 mM 2-mercaptoethanol. Apophosphorylase b was prepared by the method of Graves et al. (20). Apophosphorylase routinely used showed less than 0.1% of the activity of the native phosphorylase b. Phosphorylases reconstituted with 6-FPAL and 6-FPLP were prepared by incubating apophosphorylase with a 50-fold excess of 6-FPAL or a 5-fold excess of 6-FPLP at pH 6.8 and 30°C for 60 or 10 minutes, respectively. Then, an equal volume of saturated, neutral ammonium sulfate was added. The protein precipitate, collected by centrifugation, was dissolved in a buffer of 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.002 M EDTA, pH 6.8, and dialyzed against several changes of this buffer. The amount of 6-FPLP bound in the enzyme was determined by the general method of Baranowski et al. (21). The protein was precipitated by adding 7% trichloroacetic

acid. After centrifugation, the concentration of 6-FPLP in the supernatant fluid was determined spectrophotometrically. The molar ratio between 6-FPLP and phosphorylase monomer was 0.98. Only freshly made (i.e., within 5 days of preparation), 6-FPAL phosphorylase b, was used in this study. 6-FPLP phosphorylase was rather stable on ice and retained more than 90% of the original activity after 2 weeks. Enzymatic activity in the direction of glycogen synthesis was measured by liberation of inorganic phosphate from glucose-1-P described by Illingworth and Cori (22) or by the incorporation of [^{14}C] glucose into glycogen with the filter paper assay of Thomas et al. (23). The protein concentration was measured spectrophotometrically by using the extinction coefficient ($\varepsilon^{1\%}_{10mm}$) at 280 nm of 13.2, according to Kastenschmidt et al. (24).

NMR Studies

^{19}F NMR spectra were obtained at 282.4 MHz on a Bruker MW 300 superconducting spectrometer. A spectral width of 20,000 Hz was employed with 28 μsec (a 60° pulse) pulse width and a repetition time of 0.21 sec. D_2O (10%) was present in the buffer used for field/frequency lock, and a 1 mm capillary containing 20 mM TFA (trifluoroacetic acid) at pH 6.8 was inserted for chemical shift referencing.

^{31}P NMR spectra were obtained at 121.5 MHz with the same spectrometer. D_2O (10%) was present in the buffer as an internal deuterium lock; a pulse width of 20 μsec (a 60° pulse) and a spectral width of 6,000 Hz were used for all spectra. A 1 mm capillary containing 85% phosphoric acid was inserted for chemical shift referencing.

Fluorescence Spectra

Fluorescence measurements were carried out with a Spex spectrofluorimeter. Samples contained 2 mg/ml enzyme, 2 mM EDTA, 40 mM α-glycerophosphate, and 30 mM β-mercapto-ethanol, at pH 6.8.

RESULTS

Earlier studies of Graves et al. (25) showed that phosphorylase reconstituted with 6-fluoropyridoxal is active in the presence of phosphite. Because the kinetic parameters were not determined, initial rate studies of 6-FPAL phosphorylase were undertaken to allow a comparison of results obtained with phosphorylase reconstituted with pyridoxal (26). Double reciprocal plots of initial rates vs. concentrations of glucose-1-P in the presence of different concentrations of phosphite converged on the X-axis as obtained with pyridoxal phosphorylase b, suggesting that a sequential mechanism exists.

The kinetic parameters are summarized in Table 1 and are similar to those obtained for pyridoxal phosphorylase except that 6-FPAL phosphorylase has a lower V_M value (V_{max} value of the pyridoxal enzyme is 15.4 I.U.). Also, the effect of glucose, an allosteric inhibitor was tested with respect to glucose-1-P. Competitive kinetics were found, and a K_I value of 45 mM was evaluated, which is again close to that of pyridoxal phosphorylase (K_I = 50 mM) reported earlier (27).

Kinetic parameters of 6-FPLP reconstituted phosphorylase b also were evaluated by initial rate studies at various concentrations of glycogen and glucose-1-P and are shown in

Table 1. Kinetic parameters of 6-FPAL and 6-FPLP phosphorylase

Enzyme	Parameter
6-FPAL Enzyme	K_A = 0.8 mM $K_{A'}$ = 0.9 mM $K_{s'}$ = 15.4 mM K_S = 13.9 mM V_{max} = 4.2 μmole/min. mg
6-FPLP Enzyme	Km (glucose-1-P) = 7.4 mM Km (glycogen) = 0.037% (gm/100 ml) V_{max} = 17.8 μmole/min. mg

K_A = dissociation constant of phosphite from an enzyme-phosphite-AMP-glycogen complex

$K_{A'}$ = dissociation constant of phosphite from an enzyme-phosphite-AMP-glycogen-glucose-1-P complex

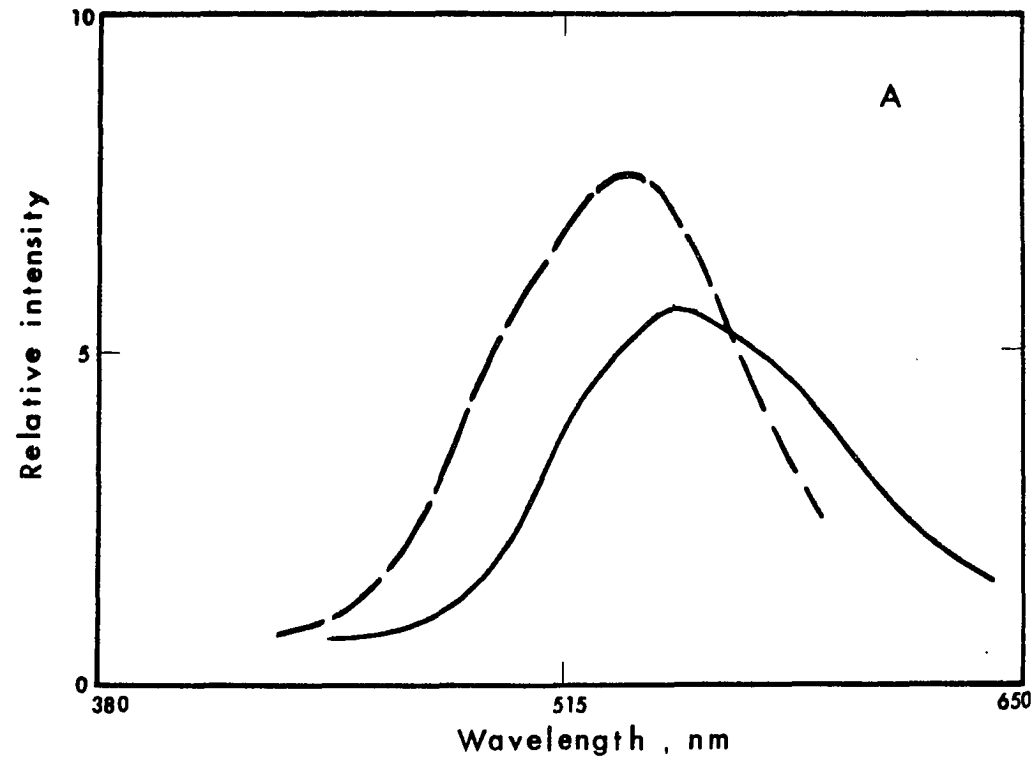
K_S = dissociation constant of glucose-1-P from an glucose-1-P-enzyme-AMP-glycogen complex

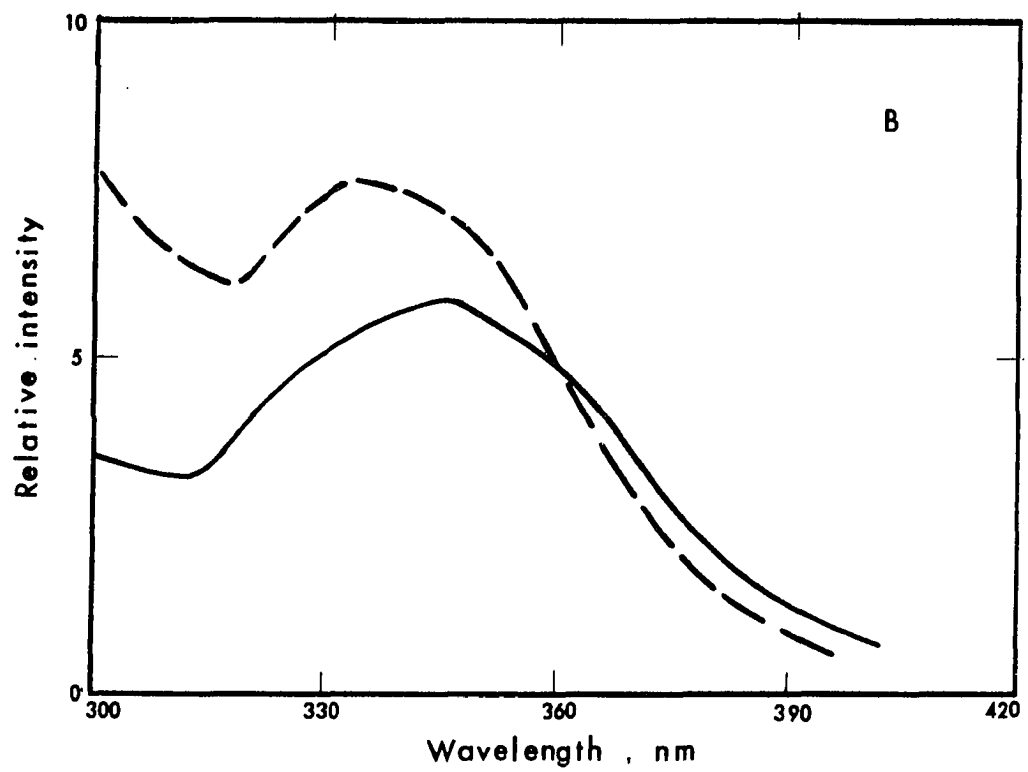
$K_{S'}$ = dissociation constant of glucose-1-P from an enzyme-AMP-glycogen-glucose-1-P-phosphite complex.

Table 1. Except for the lower V_{max} value (V_{max} value of the native phosphorylase is 64 I.U.), kinetic parameters of the 6-FPLP enzyme are almost identical to those of native phosphorylase b. When the response of 6-FPLP phosphorylase b to AMP was measured, a concave upward curve was observed in the double-reciprocal plot of 1/velocity vs. 1/[AMP]. The Hill coefficient of 1.45 was very close to the value evaluated for native phosphorylase b (28). All of the above results show that 6-FPLP and 6-FPAL reconstituted phosphorylase b retain most of the kinetic properties of the native and the pyridoxal reconstituted phosphorylase b, respectively, except for the lower V_{max} values.

To learn why the enzymatic activity of 6-FPLP phosphorylase is lower than the native enzyme, NMR, UV, and fluorescence spectroscopic measurements were done to study the enzyme-bound coenzyme. The ^{31}P NMR spectrum of the free 6-FPLP enzyme showed a signal at 0.6 ppm. When 2.5 mM AMP-S was added to the same enzyme solution, the intensity of the signal at 0.6 ppm decreased and a new peak appeared at 3.0 ppm. Because these spectral results are virtually identical to those found with the native phosphorylase (11), these studies suggest that the substitution of the 6-H atom of the enzyme-bound PLP by a fluorine atom does not change the interaction of the 5'-phosphoryl group of PLP with the

Figure 3. Fluorescence spectra of 6-FPLP reconstituted and native phosphorylase b. (A) Broken line, emission spectrum of native phosphorylase b (2.1 mg/ml), excitation wavelength at 330 nm. Solid line, emission spectrum of 6-FPLP reconstituted phosphorylase (1.5 mg/ml), excitation wavelength at 345 nm. (B) Broken line, excitation spectrum of native phosphorylase b (2.1 mg/ml), emission wavelength at 530 nm. Solid line, excitation spectrum of 6-FPLP reconstituted phosphorylase (1.5 mg/ml), emission wavelength at 550 nm


A
Relative intensity
10
5
0
380
515
650
Wavelength , nm

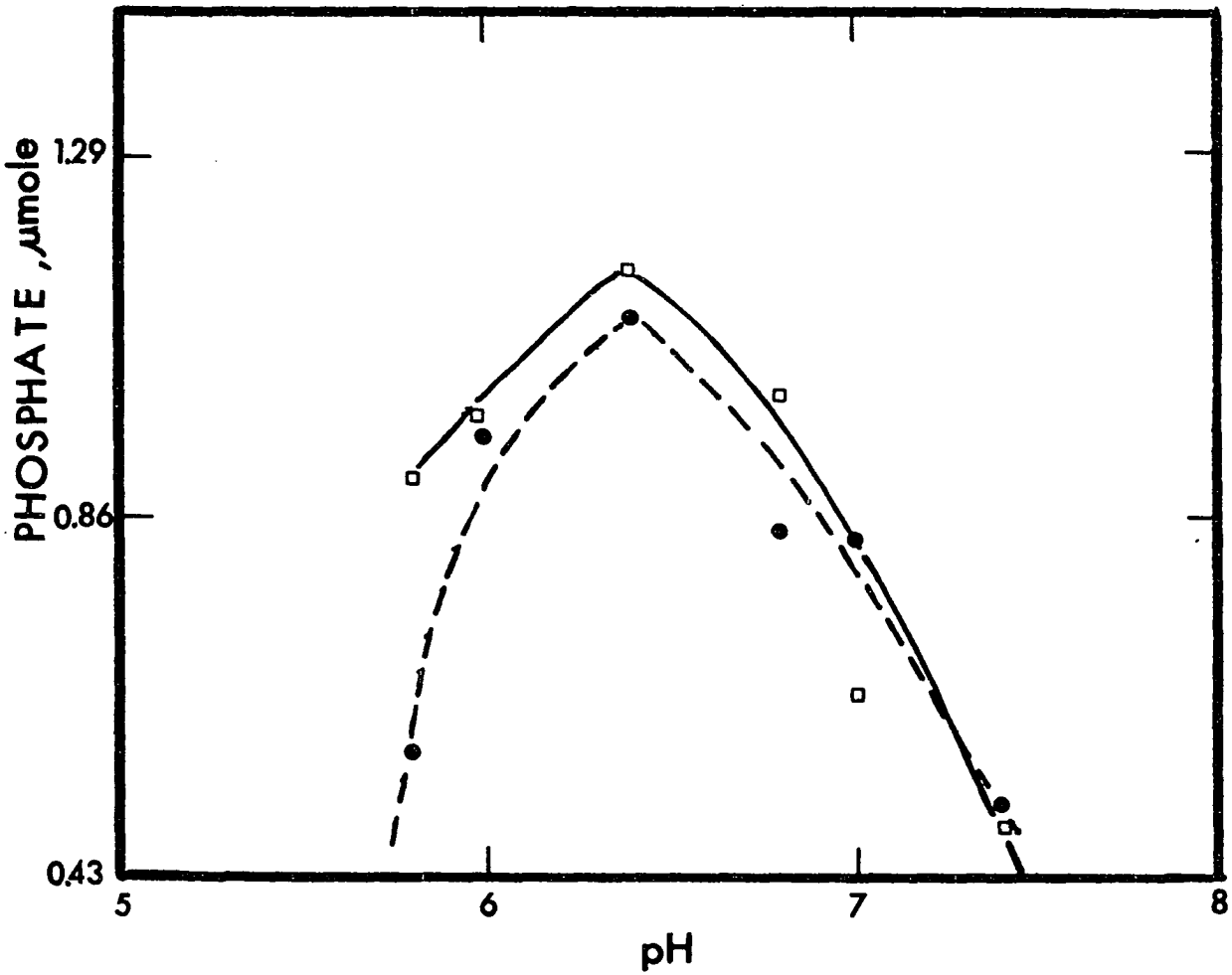

B
Relative intensity
10
5
0
300
330
360
390
420
Wavelength , nm

protein in the active and inactive states. The ionization state of the 3-OH group on the coenzyme in 6-FPLP phosphorylase can be determined by its ^{19}F NMR and UV spectra. The ^{19}F NMR spectrum of free 6-FPLP phosphorylase (Figure 5) shows a signal at -12.2 ppm, which corresponds to a 6-FPLP with a protonated 3-OH group and an unprotonated ring nitrogen, according to the pH titration curve shown in Figure 2. The UV spectrum of 6-FPLP phosphorylase shows an absorbance maximum at 343 nm which coincides with the absorbance maximum, 345 nm, of the Schiff's base of 6-FPLP with valine in the neutral enol-imine form (29). These studies show that 6-FPLP forms a neutral enol-imine Schiff's base with a protonated 3-OH and unprotonated ring nitrogen, when it is bound in phosphorylase. Fluorescence spectra of this enzyme were also taken, as shown in Figure 3. The excitation spectrum of 6-FPLP phosphorylase, with emission at 550 nm, shows a maximum at 345 nm. And the emission spectrum of this enzyme, with excitation at 343 nm, shows a maximum at 550 nm. The UV and fluorescence spectral results of 6-FPLP phosphorylase are comparable to those obtained with the native enzyme (30, 31), except all the peaks were shifted to longer wave lengths. This is likely due to the presence of a fluorine atom at the 6-position of the coenzyme which can lower the absorption energy of the coenzyme (15). Because of the spectral similarities between 6-FPLP and native

phosphorylase, these studies further confirm that PLP in native phosphorylase is bound as a neutral enol-imine Schiff's base, as suggested by Johnson et al. (32) and Gani et al. (33). The pK_a value of the 3-OH group in 6-FPLP is similar to the pK_a value determined for the deprotonation reaction of the 3-OH group on 3-hydroxypyridine with an unprotonated ring nitrogen. This compound has been used as a model to describe the microscopic dissociation constant of the same reaction in PLP. The pK_a value of the Schiff's base of 6-FPLP and valine is 12.5 (29). It is very similar to the pK_a value obtained with the Schiff's base of PLP with valine, 11.7 (34). The pK_a has been assigned to the dissociation of the hydrogen-bonded proton of the Schiff's base in which the ring nitrogen is not protonated. These comparisons clearly showed that the basicities of the 3-OH group of 6-FPLP and its Schiff's base are similar to the 3-OH groups in PLP and its Schiff's base when the PLP ring nitrogen is not protonated. Because both coenzymes in 6-FPLP and the native phosphorylases stay as enol-imine Schiff's bases, with the ring nitrogens unprotonated, the basicities of the 3-OH groups of the coenzyme in these enzymes should be similar.

Substitution of 6-H of PLP or pyridoxal by a fluorine atom dramatically changes the basicity of the pyridine nitrogen in these compounds. In order to test the possibility

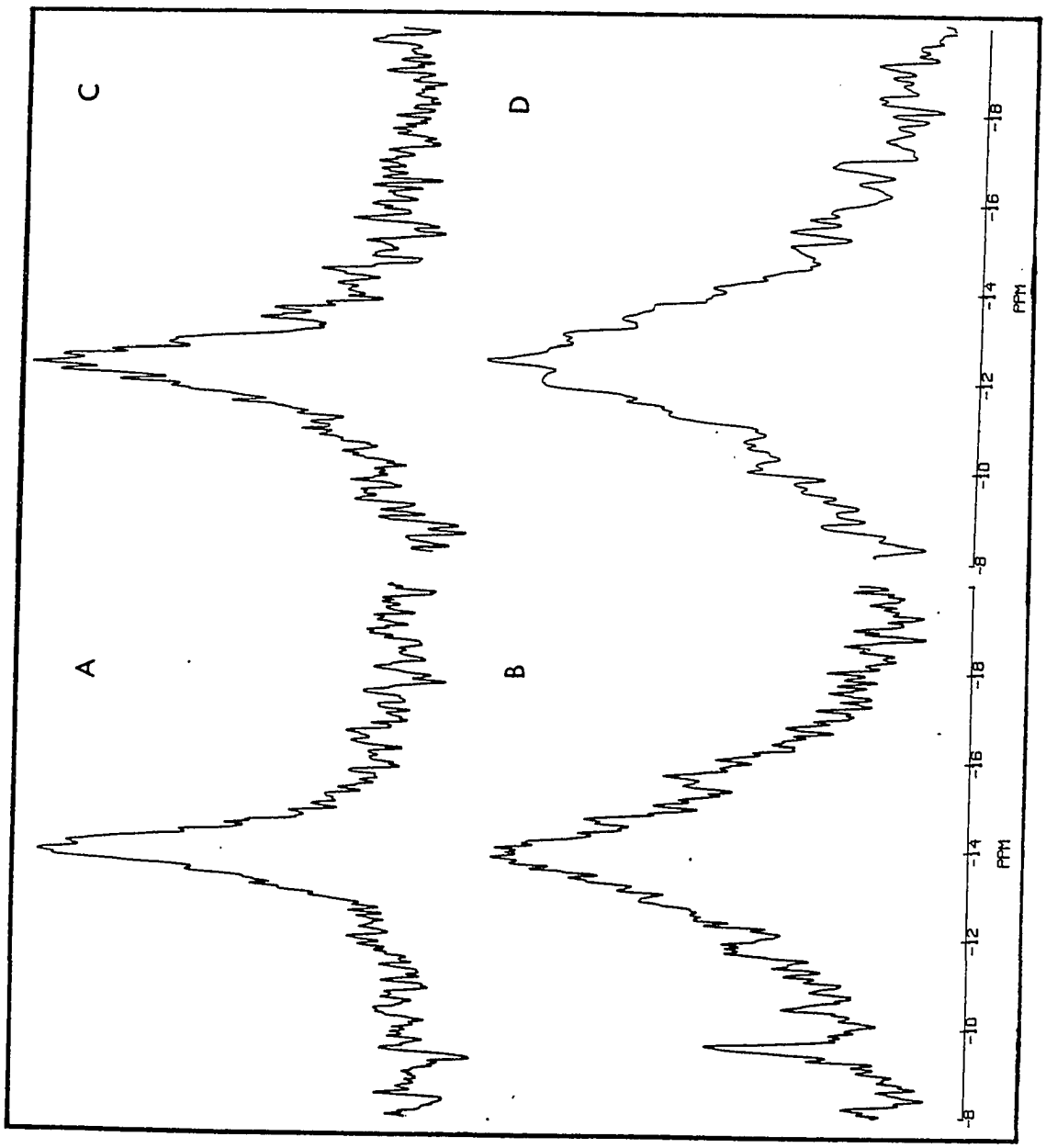
Figure 4. Enzymatic activity of 6-FPLP and native phosphorylase b at different pH values. Assay solutions at 30°C and varied pH contained 0.020 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.016 M glucose-1-P, 1% glycogen, and 11.8 µg/ml of native phosphorylase b (□) or 47.2 µg/ml of 6-FPLP phosphorylase (●). Reaction mixture was stopped at 5 mins, and inorganic phosphate concentration was determined by the method described by Illingworth and Cori (22)


PHOSPHATE ,µmole
1.29
0.86
0.43
5
6
7
8
pH

that the pyridine nitrogen of PLP is one of the determining factors of the pH dependency of the activity of phosphorylase, the enzymatic activities of 6-FPLP reconstituted and the native phosphorylases at varied pH values were assayed. No difference can be found in the resulting profiles (Figure 4). This result indicates that the pyridine nitrogen of PLP is not responsible for the pH dependency of the enzymatic activity of phosphorylase.

In order to evaluate the binding strength of the coenzymes in 6-FPLP reconstituted and native phosphorylases, the resolution and reconstitution rates of the coenzymes in these enzymes were studied. In the presence of 0.2 M cysteine and 0.2 M imidazole at pH 6.0, the coenzyme can be resolved from phosphorylase (35). 6-FPLP reconstituted phosphorylase b lost its activity gradually under these conditions and no difference could be found in its rate of inactivation from that of the native phosphorylase (results not illustrated). The rates of the reconstitution reactions of apophosphorylase with 6-FPLP or with PLP, determined by the restoration of the enzymatic activities, are virtually identical (results not illustrated). These results indicates that 6-FPLP binds to phosphorylase as tightly as PLP does. ^{19}F NMR spectra of 6-FPAL and 6-FPLP reconstituted phosphorylase in the absence and presence of substrates and

Figure 5. ^{19}F NMR spectra of phosphorylase reconstituted with 6-FPAL and 6-FPLP. (A) Free 6-FPAL enzyme (26 mg/ml), 21,000 acquisitions and LB = 20 Hz. (B) 6-FPAL enzyme (25.4 mg/ml) in the presence of AMP (1 mM), maltopentaose (1%), and glucose-1-P (20 mM), 33,000 acquisitions and LB = 20 Hz. (C) Free 6-FPLP phosphorylase (28 mg/ml), 23,000 acquisitions and LB = 20 Hz. (D) 6-FPLP enzyme (26 mg/ml) in the presence of AMP (1 mM), maltopentaose (1%), and glucose-1-P (20 mM), 43,000 acquisitions and LB = 30 Hz


A
B
C
D
-8
-10
-12
-14
-16
-18
PPM

effectors were taken and shown in Figure 5. The presence of ligands broadened the ^{19}F resonances of these proteins, but their chemical shifts were not changed.

DISCUSSION

^{19}F NMR study of 6-FPAL and 6-FPLP showed that the chemical shifts of these compounds are sensitive to the ionization state of the 3-phenolic groups. A study of 6-fluoropyridoxine (36) indicates that the protonation of the pyridine nitrogen, at pH<1, can also shift the ^{19}F NMR signal up-field. On the basis of ^{19}F NMR spectra of 6-FPLP in the solutions containing varied amounts of ethylene glycol, we have found that the change of the hydrophobicity of solution did not affect the chemical shift of ^{19}F resonances of 6-FPLP and 6-FPAL. Therefore, the chemical shift of the ^{19}F signals of either free or enzyme-bound 6-FPAL and 6-FPLP can be used as an indicator of the ionization states of the pyridine nitrogen and 3-phenolic group. ^{19}F NMR spectra of phosphorylases reconstituted with 6-FPAL and 6-FPLP show that the chemical shift of these ^{19}F resonances did not change when substrates and activators were added. The results indicate that the ionization states of the pyridine nitrogen and 3-phenolic groups of the coenzymes in these enzymes were not affected by the catalytic process. Because the basicity of the 3-phenol group of the enzyme-bound 6-FPLP is similar to the basicity of the same group in the native phosphorylase, this result suggests that the dissociable proton of the 3-phenolic group of PLP is not involved in any acid-base reaction essential for the catalysis

of phosphorylase, and it is consistent with the study of phosphorylase reconstituted with O-methyl pyridoxal phosphate (12).

The studies of phosphorylase reconstituted with 6-FPLP show that the substitution of the 6-H of PLP by a fluorine atom does not affect most of the properties of phosphorylase. 6-FPLP reconstituted phosphorylase shows similar pH and temperature dependencies of enzymatic activity, dissociation constants of ligands, and tightness of coenzyme binding to those of the native phosphorylase. Even the interaction between the 5'-phosphoryl group of the coenzyme with protein and the basicity of the 3-phenolic group of the coenzyme in 6-FPLP reconstituted and native phosphorylase are very similar. However, 6-FPLP reconstituted was found less active than the native phosphorylase, and the basicity of the pyridine nitrogen of the coenzyme in the former enzyme is significantly lower than that in the native phosphorylase. Therefore, the lower activity is likely due to a change of the interaction between the pyridine nitrogen with some residue on the enzyme.

By examining the X-ray crystallographic map of phosphorylase in the "T" state (7), the only functional group within the range of 5Å of the ring nitrogen of PLP is the carboxyl group of Glu 645. But the closest distance between

this nitrogen with one of oxygens of this carboxyl group is 4.5Å which is too far to allow the formation of a strong H-bond. Because various studies (27, 37, 38) showed that the protein structure of phosphorylase in the "R" state is significantly different from that of phosphorylase in "T" state, it is likely that the relative position of the pyridine nitrogen of PLP and the carboxyl group of Glu 645 may change and can interact strongly during catalysis. It is supported by the observation of Bresler and Firsov (39). On the basis of differential spectrophotometric studies of glycogen phosphorylase, these authors suggested that the pyridine nitrogen of PLP is protonated when the enzyme forms a Michaelis complex with the substrate, although this explanation is still in controversy (40). Although the pK_a value of the ring nitrogen of PLP in aqueous solution is 8.7 (41), it can be significantly lowered when it locates in a hydrophobic environment and the 3-phenolic group is protonated (34). Therefore, it can accept a proton and form a H-bond with the protein. That a carboxyl group with a high pK_a value is important to the catalysis of phosphorylase is also suggested by the chemical modification study of phosphorylase with water-soluble carbodiimide (42).

Although this study indicates that the pyridine nitrogen of the enzyme-bound PLP may form a H-bond with the carboxyl

group of Glu 645 in the active phosphorylase, the role of this interaction during the process of catalysis is still unclear. However, because the location of the ring nitrogen of the coenzyme is distant from the glucose-1-P binding site in phosphorylase, a charge-relay system connecting them seems unlikely. That the dramatic change of the basicity of the ring nitrogen did not totally abolish the catalytic capability of phosphorylase suggests that the interaction between the pyridine nitrogen of PLP and the protein is not essential for the catalysis of phosphorylase. Therefore, the role of the ring nitrogen of PLP in phosphorylase is likely to be structural, rather than catalytic. A possible function of this pyridine nitrogen is forming a contact with the protein in the activated phosphorylase to keep the co-enzyme in a certain orientation which can facilitate the catalysis of phosphorylase.

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SECTION 3: ^{19}F NMR STUDIES OF PHOSPHORYLASE RECONSTITUTED WITH 6-FLUOROPYRIDOXAL AND 6-FLUOROPYRIDOXAL PHOSPHATE

ABSTRACT

^{19}F NMR spectra of phosphorylase reconstituted with 6-fluoropyridoxal phosphate (6-FPLP) and 6-fluoropyridoxal (6-FPAL) in the absence and presence of various ligands were obtained. The ^{19}F resonances of proteins containing 6-FPLP or 6-FPAL in the activated state, in the presence of all substrates and effectors, are significantly broader than those of the free enzymes. In order to interpret the significance of these spectral changes, nuclear relaxation mechanisms of the fluorine nucleus were studied. Analysis of the ^{19}F NMR spectra of 6-FPLP enzyme obtained with two NMR spectrometers, with magnetic field of 90 and 300 MHz, indicates that both the dipole-dipole interactions and chemical shift anisotropy of the fluorine nucleus in the activated enzyme are greater than those in free protein. Spin-lattice relaxation times (T_1) of 6-FPLP and 6-FPAL reconstituted enzymes were also measured. Nuclear Overhauser effects of free 6-fluoropyridoxine, 6-FPLP, and 6-FPAL at varied irradiation frequency indicate the existence of an intramolecular, dipole-dipole interaction between the 6-fluorine and the 5'-protons in these compounds. These studies suggest that the protein structures of free and activated phosphorylases are quite different. When phosphorylase is in the activated state, the pyridine ring of PLP is bound more tightly to the enzyme

and the configuration of the coenzyme molecule may be changed, probably the relative position between the 5'-hydrogens and 6-hydrogen (fluorine) is altered.

INTRODUCTION

Kinetic studies of glycogen phosphorylase b indicate that its enzymatic activity is controlled by an equilibrium between at least two conformations, an inactive "T" conformer and an activated "R" conformer (1,2). The equilibrium is determined by the binding of various ligands to the protein, such as AMP, glucose-1-P, and orthophosphate which can stabilize the "R" conformer, and glucose, caffeine, and glucose-6-P which can stabilize the "T" conformer. Heterotropic allosteric interactions among the binding of ligands to phorphorylase have also been observed (3, 4, 5). X-ray crystallographic studies of phosphorylase in the presence of glucose-1-P (6) or glucose cyclic 1,2-phosphate (7) which is a potent competitive inhibitor with respect to glucose-1-P, show that the protein undergoes substantial structural changes upon the binding of these ligands. However, a protein structure of a fully activated phosphorylase carrying out catalysis has not been determined yet.

The possibility that the coenzyme, PLP, is a prerequisite for the allosteric transition of phosphorylase have been tested by studying the cooperative binding of ligand to phosphorylase reconstituted with different PLP analogues. Phosphorylase reconstituted with pyridoxal (8), 5'-deoxypyridoxal (9), or pyridoxal phosphate monomethyl ester (10),

which are completely inactive, still bind AMP in a highly cooperative manner. These results, along with the tritium-hydrogen exchange study of pyridoxal phosphate monomethyl ester reconstituted phosphorylase (10), indicate that at least the 5'-phosphoryl group of PLP is not needed for the allosteric transition of phosphorylase. The possibility that the rest of the coenzyme molecule is involved in the conformation transitions of phosphorylase, however, can not be ruled out.

Because phosphorylase reconstituted with 6-FPLP and 6-FPAL retain most of the structural characteristics of the native and pyridoxal reconstituted phosphorylase, including the cooperative binding of AMP and glucose (11), we have used these reconstituted enzymes as a model system to study the conformational changes around the PLP binding site in phosphorylase. NMR spectroscopic techniques has been widely used in studying the conformational properties of biological macromolecules, such as oligopeptides, DNA, RNA, and proteins (12). Analysis of the nuclear relaxation mechanisms of these samples have yielded fruitful information about the mobilities or the orientations of different residues inside the whole molecule (13, 14). In this study, nuclear relaxation mechanisms of the fluorine nucleus in the 6-FPLP and 6-FPAL reconstituted phosphorylases have been investigated,

and the results provide more insight about binding and interactions of the coenzyme inside phosphorylase.

METHODS AND MATERIALS

The chemicals, proteins, conditions of ^{19}F NMR measurements by the Bruker WM 300 spectrometer, and enzymatic activity assay used have been described in the preceding paper. ^{19}F NMR spectra of 6-FPLP reconstituted phosphorylase were also obtained with a Bruker HX90 at Iowa State University. A 60° pulse angle was applied, and the recycle time for the experiment was 0.5 sec. T_1 values of samples were measured with Bruker MW 300 spectrometer by using the 180°- -90° pulse method, and the Bruker software was used to analyze the resulting recovery curves. Because long accumulation times, about 30 hours were required to obtain a T_1 value from protein samples, a modified fourier transform computer program that can acquire FID's in a cyclic fashion, as described by Hull and Sykes (15), was adopted to avoid any systematic error due to long-term fluctuation in the instrument. Specific enzymatic activity of the sample was assayed before and after these long-term experiments to make sure the enzyme was not denatured.

For NOE experiments, a selective radio frequency pulse was applied for 10 seconds, followed by the observation pulse. A 10-second delay was inserted between the observation pulse and the next preirradiation if more than one transient was

required to get accurate measurement of the intensity. The selective irradiation was applied over a wide spectral range, covering the proton resonances of the coenzyme. The changes in intensity were measured from changes in the integrated intensity.

RESULTS

^{19}F NMR Studies of 6-FPAL Phosphorylase

^{19}F NMR spectra were obtained for phosphorylase reconstituted with 6-FPAL in the presence and absence of different ligands, as shown in Table 1. The 6-FPAL enzyme showed a resonance at -14.1 ppm from the external standard, TFA, with a line-width of 320 Hz. The difference between the chemical shift of the enzyme-bound 6-FPAL and the value measured from its pH titration curve (Figure 2, in the preceding paper), -9 ppm, can be explained by their structural differences in that the 4-aldehyde group of free 6-FPAL is a hemiacetal while it forms a Schiff's base with the ε-amino group of a lysyl residue when it is bound to the enzyme. When 1 mM AMP was included in the protein solution, 6-FPAL phosphorylase showed a resonance at -13.9 ppm with a line-width of 470 Hz. That the line-width of this signal is significantly broader than that of the free enzyme can be explained by the fact that in the presence of AMP phosphorylase b dimer (MW = 200,000) associates into a tetramer (MW = 400,000) (16). The latter enzyme form has longer rotational correlational time (17) and thus wider signal than the former enzyme form. The effect of phosphite, an activator, on the ^{19}F NMR spectrum of the enzyme-bound 6-FPAL was also investigated. With 5 mM phosphite, the

Table 1. ^{19}F NMR spectra of 6-FPAL reconstituted phosphorylase

Condition	Chemical shift, (ppm)	Half-height width, (Hz)
Free enzyme	-14.1	330
+1 mM AMP	-13.9	480
+5 mM phosphite	-13.7	570
+5 mM phosphite +70 mM glucose	-13.8	360
+5 mM phosphite +1 mM AMP	-13.8	540
+1 mM AMP +5 mM phosphite +16 mM glucose-1-P	-14 -15.5	
+1 mM AMP +5 mM phosphite +16 mM glucose-1-P +1% maltopentaose	-14	760

6-FPAL enzyme showed a resonance at -13.7 ppm and a line-width of 550 Hz. This broad line-width indicates that 6-FPAL phosphorylase is in a tetrameric state under this condition. Glucose (70 mM), an allosteric inhibitor of phosphorylase b, which can dissociate the tetrameric pyridoxal phosphorylase b into dimers (16) reduces the line width of the 6-FPAL enzyme containing 1 mM AMP. Adding glucose-1-P (20 mM) to the mixture of 6-FPAL enzyme, phosphite (5 mM), and AMP (1 mM) produced a second resonance at -15.5 ppm in addition to the original signal at -14.1 ppm. This spectrum indicates the presence of two species of the 6-FPAL enzyme with similar concentrations in solution. Because the concentration of glucose-1-P used in this sample is close to its K_m value, 22 mM, for the 6-FPAL enzyme, approximately one-half of the 6-FPAL enzyme might be expected to form a complex with glucose-1-P, and, therefore, the new peak at -15.5 ppm can be assigned to an enzyme-phosphite-glucose-1-P-AMP complex. Because two distinct peaks are seen in this spectrum, it is evident that the exchanging process between free and bound glucose-1-P is slow (18). The observed difference in the chemical shift indicate the ionization states of the functional groups of enzyme-bound 6-FPAL in the glucose-1-P bound complex are different from that in the free enzyme. In the presence of AMP (1 mM), glucose-1-P (20 mM), maltopentoase (catalytically active under this condition) showed

Table 2. ^{19}F NMR spectra of 6-FPLP reconstituted phosphorylase

Condition	Chemical shift (ppm)	Half-height width, (Hz)
Free enzyme	-12.3	350
+1 mM AMP	-12.2	590
+1 mM AMP +20 mM glucose	-11.8	360
+1 mM AMP +16 mM glucose-1-P	-13.8	560
+1 mM AMP +16 mM glucose-1-P +1% maltopentaose	-12.4	760
+1% maltopentaose	-12.4	410
+1% maltopentaose +1 mM AMP	-11.6	650
+1% maltopentaose +1 mM AMP +9 mM glucose cyclic 1,2-phosphate	-12.3	360

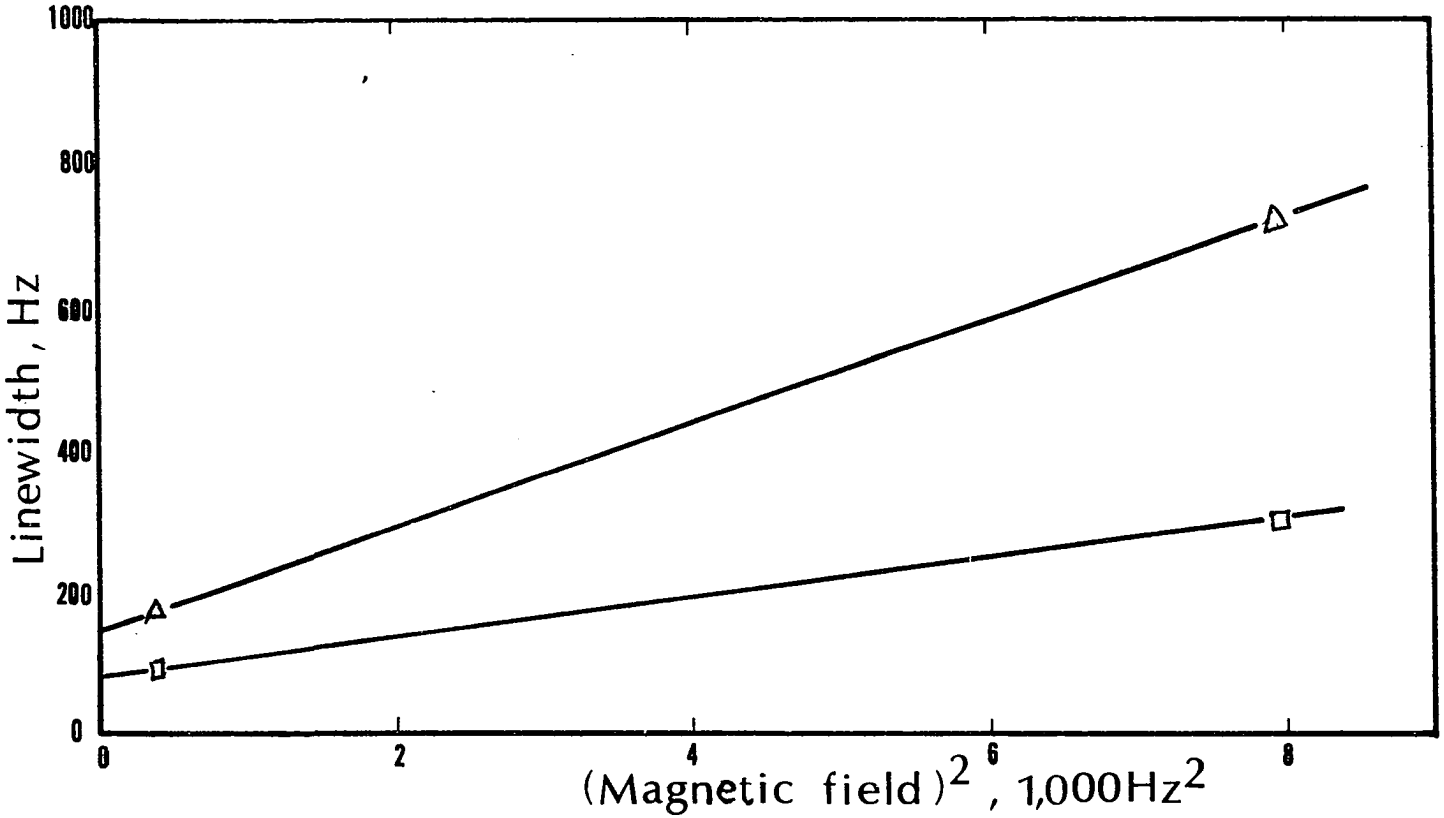
a resonance at -13.8 ppm with a line-width of 770 Hz. Various studies have shown that phosphorylase b and pyridoxal reconstituted phosphorylase b in the activated state are dimers (3, 5). That the line-width of the activated 6-FPAL enzyme is broader than that assumed for the tetrameric species, around 550 Hz, implies that a change occurs around the coenzyme binding site when the enzyme is carrying out the catalytic reaction.

^{19}F NMR Studies of 6-FPLP Phosphorylase

^{19}F NMR spectra also were obtained for phosphorylase b reconstituted with 6-FPLP and are shown in Table 2. The ^{19}F NMR spectra of the free and activated 6-FPLP enzymes have already been shown in the Figure 5 of the preceding paper. When the positive effector, AMP, (1 mM) was added to the free enzyme solution, the line-width of the resulting ^{19}F signal was broadened to 590 Hz, but the chemical shift was not affected. When glucose (20 mM) was included in the sample containing enzyme and AMP (1 mM), the 6-FPLP enzyme showed a resonance at -11.8 ppm with the line-width of 360 Hz. The variation of the line-widths of the ^{19}F signals in these samples also clearly demonstrates a correlation between the line-width and the polymerization state of the 6-FPLP enzyme. When maltopentaose (1%) was added, the 6-FPLP enzyme showed a resonance at -12.4 ppm with a line-width of 440 Hz. In the

presence of AMP (1 mM) and maltopentaose (1%), the 6-FPLP enzyme showed a ^{19}F signal at -11.6 ppm with the line-width of 650 Hz. That the line-widths of the resonances in the presence of maltopentaose are observed to be wider than those without maltopentaose implies that the presence of maltopentaose can either increase the molecular size of 6-FPLP enzyme or change the environment around the enzyme-bound 6-FPLP. However, it is not likely that maltopentaose will cause tetramer formation because it is known that oligasaccharides favor the dissociation of phosphorylase from the tetrameric to the dimeric state (5). Upon adding glucose-1-P (16 mM) and AMP (1 mM), the ^{19}F signal of 6-FPLP enzyme was observed to move 1.5 ppm downfield, an effect similar to that found for the binding of glucose-1-P to 6-FPAL enzyme. In the presence of the substrates, glucose-1-P (16 mM) and maltopentaose (1%), and the effector, AMP (1 mM), the activated 6-FPLP phosphorylase b showed a resonance at -12.4 ppm with a line-width of 760 Hz. When one of the substrates in the above sample, glucose-1-P, was replaced by an inhibitor, glucose cyclic 1,2-phosphate (9 mM), the resulting ^{19}F NMR spectrum showed a resonance at -12.3 ppm with a line-width of 360 Hz corresponding to that of a dimeric species.

Figure 1. ^{19}F NMR half-height widths for phosphorylase reconstituted with 6-FPLP as a function of the square of the spectrometer frequency at 293°K. 6-FPLP enzyme was dissolved in buffer solution (40 mM β-glycerophosphate, 30 mM 2-mercaptoethanol, and 2 mM EDTA, at pH 6.8) either as free protein or as a complex with substrates. (△) free 6-FPLP phosphorylase (40 mg/ml). (□) 6-FPLP phosphorylase (40 mg/ml) in the presence of malotpentaose (1%), AMP (1 mM), and glucose-1-P (20 mM). Line-widths of these ^{19}F resonances were corrected by substrating the line broadening factor used from experimental data


1000
800
600
400
200
0
Linewidth, Hz
0
2
4
6
8
(Magnetic field)^2, 1,000Hz^2

Field Dependency of the Line-width of 6-FPLP Phosphorylase

Several relaxation mechanisms may contribute to the nuclear relaxation rates. Since fluorine is covalently linked to 6-FPLP reconstituted phosphorylase, which has a rotational correlation time estimated to be longer than 70 nsec (17), the spin rotation and scalar relaxations can be neglected, for these mechanisms are usually important for molecules with short correlation time. Dipole-dipole interactions of the fluorine nucleus with the neighboring spins and chemical shift anisotropy, however, could be important relaxation mechanisms. It has been demonstrated that the contributuions of the line-width of a NMR signal due to the former interaction is independent to the magnetic field of the NMR spectrometer (19) and is proportional to γ_{ij}^{-6} where γ_{ij} is the internuclear vector and i and j represent the interacting nuclei (20). The contributions of the line-width due to chemical shift anisotropy is a function of the square of the magnetic field (19). Therefore, to distinguish line-width contributions due to these two mechanisms, ^{19}F NMR spectra of free and activated 6-FPLP reconstituted phosphorylase were measured at two different magnetic fields, 84.7, and 282.4 MHz. The line-width values of the ^{19}F resonances in these spectra were plotted against the square of the magnetic field

Table 3. Spin-lattice relaxation time (T_i) of 6-FPAL and 6-FPLP reconstituted phosphorylase

Enzyme	Condition	T_1 (sec)	Standard error (sec)
6-FPAL enzyme	Free enzyme	0.8[a]	<0.1
	+Phosphite (5 mM) + AMP (1 mM) +Glucose-1-P (20 mM) +Maltopentaose (1%)	0.8[a]	<0.1
6-FPLP enzyme	Free enzyme	1.1[b]	0.1
	+AMP (1 mM) + Glucose-1-P (20 mM) + Maltopentaose (1%)	0.8[b]	0.1
	+AMP (1 mM) + Glucose 1,2-cyclic phosphate (9 mM) + Maltopentaose (1%)	1.1[a]	0.1

[a]Mean of two independent experiments.

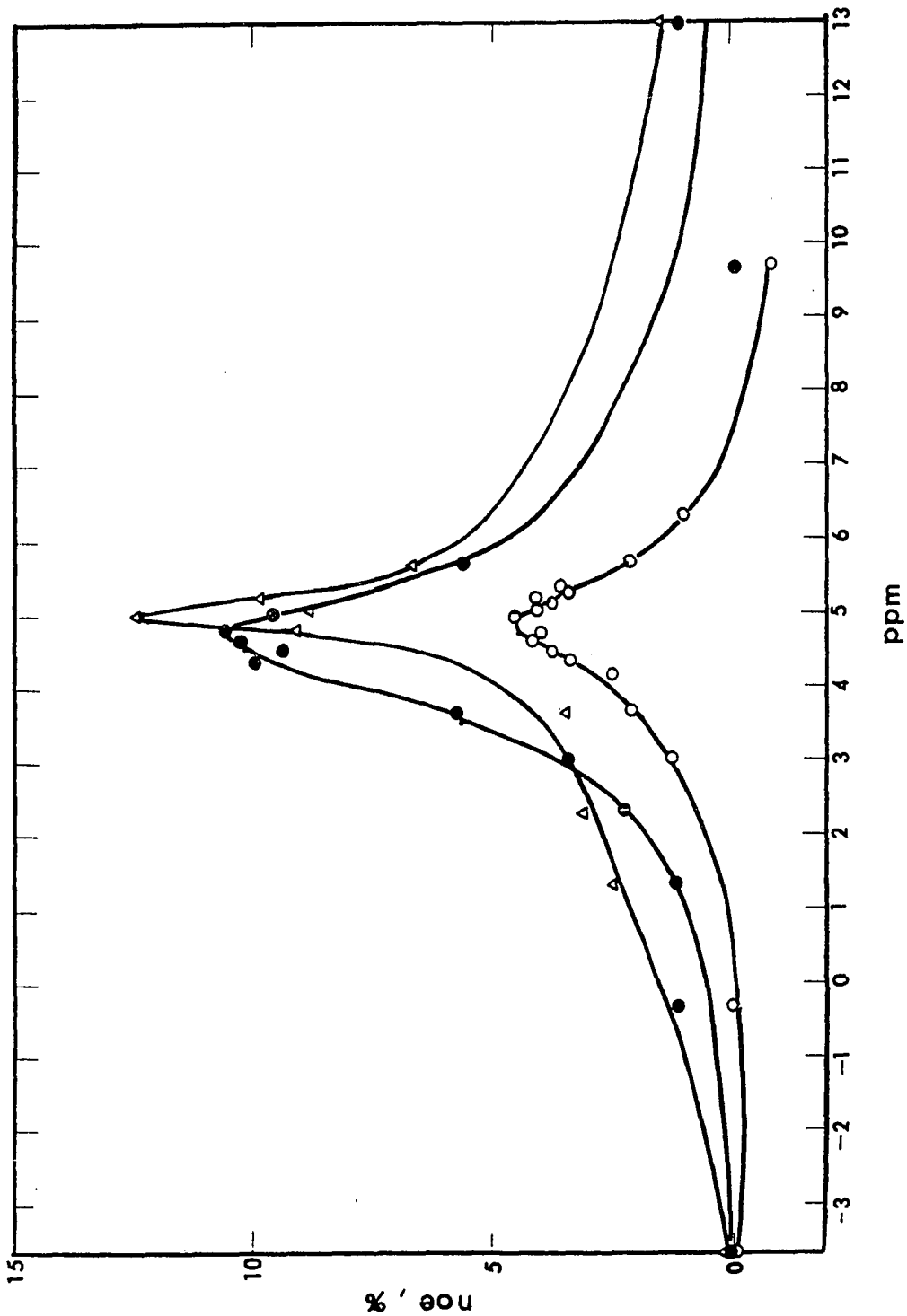
[b]Mean of three independent experiments.

applied in the spectrum, as shown in Figure 1. The y-intercepts in this figure represent the line-width contribution due to the dipole-dipole interactions, and the slopes of the lines indicate the contribution due to chemical shift anisotropy. According to Figure 1, both the dipole-dipole interactions and chemical shift anisotropy of the fluorine nucleus in the activated enzyme are stronger than those in the free enzyme.

T_1 Values of Phosphorylase Reconstituted with 6-FPAL and 6-FPLP, and T_1 and NOE of Free Fluorinated Analogues

The spin-lattice relaxation rate, $1/T_1$, is a measure of the dissipation of energy from the target nucleus to its surroundings; this rate also reflects the environment around the target nucleus and the dynamic features of the whole molecule. Therefore, this parameter was evaluated with free fluorinated analogues and the derived phosphorylases to learn about the structure around the coenzyme binding site and the role of the coenzyme in phosphorylase. T_1 values of phosphorylase reconstituted with 6-FPAL and 6-FPLP (0.5 mM) have been measured, shown in Table 3. The recovery curves of these measurements were exponential, with the standard deviation lower than 10%. It is evident that there is a significant decrease in T_1 as one proceeds from free or inhibited, to

Figure 2. Nuclear Overhauser Enhancement effects of 6-FPAL, 6-FPLP, and 6-fluoropyridoxine. NOE effects of 6-FPAL (O), 6-FPLP ( ), and 6-fluoropyridoxine (●) were measured at various 'H irradiation frequency. NOE is defined as $\frac{I-I_o}{I_o}$, where I and I_o are the ^{19}F resonance intensities of on-resonance and off-resonance 'H irradiations, respectively. The 'H NMR signals of the 5-methylene protons in 6-FPAL, 6-FPLP, and 6-fluoropyridoxine are at 5.08 ppm, 5.21 ppm, and 4.80 ppm, respectively


noe , %
15
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5
0
ppm
13
12
11
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9
8
7
6
5
4
3
2
1
0
-1
-2
-3

Table 4. Spin-lattice relaxation time (T_1) of fluorinated derivatives

Compounds	T_1 (sec)
6-Fluorpyridoxine	1.34
6-Fluorpyridoxal	2.08
6-FPLP	1.32

activated 6-FPLP reconstituted phosphorylase. But no change of T_1 values was observed between a free and an activated 6-FPAL phosphorylase. These results indicate that the spin-lattice relaxation mechanism in 6-FPLP enzyme, but not in 6-FPAL enzyme, was altered when the enzyme was transformed from an inactive state to an activated state.

T_1 values of free 6-FPAL, 6-FPLP, and 6-fluoropyridoxine have also been evaluated, shown in Table 4. Because nuclear Overhauser effects have been used to establish spatial proximity of pairs of nuclei in complex molecules (21), the NOE of the ^{19}F signal of 6-FPAL, 6-FPLP, and 6-fluoropyridoxine was studied at varying decoupling frequencies, from -3.65 ppm to 13 ppm from a trimethyl silane standard, to test the possibility that an intramolecular dipole-dipole hetero-nuclear (^1H-^{19}F) relaxation mechanism exists. All three compounds have positive NOE's (Figure 2) and show maximal NOE's near the frequency where the ^1H NMR signals of the 5'-protons were found. This result indicates the existence of a dipole-dipole relaxation mechanism of the 6-fluorine by the 5'-protons in these compounds.

DISCUSSION

^{19}F NMR studies of phosphorylase reconstituted with 6-FPAL and 6-FPLP show that the ^{19}F resonances of the activated enzymes, in the presence of substrates and effectors, are significantly broader than those of the free proteins, although the chemical shifts of these signals are not changed. Because these line-width changes can not simply be explained by the increase of the molecular size which could be due to the association of protein monomers, this observation indicates that the environments of the enzyme-bound 6-FPAL or 6-FPLP in the free and activated phosphorylases are different.

On the basis of the varied field experiment of Figure 1, the increase of both the chemical shift anisotropy and dipole-dipole interactions contribute to the broadened resonance of the activated 6-FPLP reconstituted phosphorylase. According to the theoretical studies of the nuclear relaxation rates in proteins (19), the increase of chemical shift anisotropy of the activated protein is likely due to that the coenzyme becomes less mobile when the enzyme is transformed from an inactive state into the activated state. Although the increase of the field dependent part of the line-width of the activate 6-FPLP protein could also be explained by an exchange-broadened average of the signals of free enzyme and another

activated species (19), two facts tend to negate this explanation. First, for an exchanging process the resulting chemical shift should be the weight-average of the signals corresponding to the exchanging free and activated proteins, and should be different from the chemical shift of either of these species. But no difference was detected between the chemical shifts of the activated and free 6-FPLP enzymes. Second, the ^{19}F NMR resonance of 6-FPLP reconstituted phosphorylase in the presence of substrates, AMP, and 20% ethylene glycol, which is active at 20°C, did not show any sight of splitting into different signals even at the temperature of -10°C (22). Therefore, the more likely interpretation of the broadened resonance of activated 6-FPLP enzyme is that in the activated state the coenzyme is bound more tightly to phosphorylase than in the free enzyme.

The dipole-dipole interactions of the fluorine nucleus with its neighboring spins in the activated 6-FPLP enzyme is also stronger than those in the free enzyme (Figure 1). It is consistent with the decrease of the T_1 values of 6-FPLP enzyme from an inactive state to an activated state, because dipole-dipole interaction is also a major mechanism of the spin-lattice relaxation rate. A comparison of the T_1 values of 6-FPLP reconstituted phosphorylase with those of 6-FPAL enzyme indicates the interpretation of the change of dipole-

dipole interactions is rather complex. These two enzymes, 6-FPAL and 6-FPLP phosphorylases, are expected to have similar structures, because these enzymes have structural characteristics similar to those of pyridoxal reconstituted and native phosphorylases (11) which are themselves similar in their structures (8). The virtually identical line-widths of ^{19}F resonances of 6-FPAL and 6-FPLP enzymes also support their structural similarity. Because the change of the line-widths of the free and activated 6-FPAL protein is identical to that of 6-FPLP enzyme, it suggests that certain conformational rearrangements which make coenzyme less mobile in the activated 6-FPLP enzyme may also happen in the activated 6-FPAL protein. However, the T_1 values of free and activated 6-FPAL enzyme were identical, while the T_1 of free 6-FPLP phosphorylase is longer than that of the activated 6-FPLP enzyme. Therefore, the protein structural change around the coenzyme binding site may not be the sole reason for the different T_1 values of free and activated 6-FPLP phosphorylase.

The NOE study of free 6-FPLP and 6-FPAL reveals the existence of an intramolecular dipole-dipole interaction between the 5'-protons and 6-fluorine nucleus in these compounds. If the coenzyme in free 6-FPLP reconstituted phosphorylase resembles the configuration of PLP in the phosphorylase at the partially activated state (23), the distances

between the 5'-protons and the 6-fluorine are estimated to be longer than 4Å. At this distance, the dipole-dipole interactions between the 6-F and the 5'-H's of the enzyme bound 6-FPLP should not have a significant contribution to the total relaxation rate of the fluorine nucleus in the free 6-FPLP reconstituted phosphorylase (21, 24). On the other hand, for 6-FPAL reconstituted phosphorylase, because there is no covalently bound phosphate at the 5'-position of the coenzyme to restrain the rotation along the bond connecting the pyridine ring and the 5'-carbon, the two 5'-protons can reside closely to the 6-fluorine nucleus, within 2.5Å, as in the free fluorinated compounds allowing strong dipole-dipole interactions to happen between them. Because the fluorine in the 6-FPAL enzyme, but not in 6-FPLP enzyme, has this relaxation mechanism, the T_1 value of free 6-FPAL enzyme, 0.8 sec, is shorter than that of free 6-FPLP enzyme, 1.1 sec. When 6-FPLP phosphorylase is activated, its T_1 value is shortened to 0.8 sec, similar to that of the activated 6-FPAL enzyme, suggests the configuration of the enzyme-bound 6-FPLP may be changed to allow the 6-fluorine to interact with the 5'-protons spin and hence to increase its relaxation rate. This new configuration of 6-FPLP may be achieved by a certain rotation around the pyridine - 5'-carbon bond during the transformation of phosphorylase from an inactive state to an activated state. When a bulky methyl group was introduced in PLP at either the

5'- or the 6-position, rotation around the 5-methylene group in these compounds become more difficult than in PLP and could explain why phoshorylase reconstituted with these PLP analogues showed no or very low activity (7, 25).

On the basis of these results, it is found that both the protein conformation and PLP configuration in an inactive enzyme are altered when substrates and AMP are added. And the covalent linkage between the 5'-carbon and the phosphate in the coenzyme seems important to the complete transformation of a free phosphorylase into an activated enzyme. The absence of this covalent bonding in 6-FPAL enzyme does not prevent the coenzyme from being tightly bound to the protein, as shown by the broadened ^{19}F resonance, but a change of the coenzyme configuration, implied by the unchanged T_1 values, does not occur in response to the conformational change around it. Because the V_{max} of 6-FPAL enzyme, 4.2 I.U., is lower than the V_{max} of 6-FPLP enzyme, 17 I.U. (11), the covalent bonding between the pyridine ring and the 5'-phosphoryl group in the coenzyme molecule also seems important to the catalysis. Therefore, the function of PLP molecule during the transformation of an inactive enzyme to an activated one may be that its pyridine ring can be tightly bound and change its orientation in the enzyme in response to some conformational change around it caused by the binding of substrates and AMP. This change, then, can be communicated by way of

the $-CH_2-O-\underline{P}$ group at the 5-position of PLP to the active site region. During this process, the relative orientation of the pyridine and the 5'-phosphoryl group is somehow twisted.

The present report reconfirmed that PLP plays an important structural role in phosphorylase. And because the protein structure undergoes substantial changes around PLP binding site during the "T" to "R" state transformation, the relative position between the 5'-phosphoryl group of PLP and the enzyme-bound glucose-1-P may also be changed to allow catalysis to occur.

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GENERAL DISCUSSION

Glycogen phosphorylase has been studied for over forty years. Our understanding of this enzyme, ranging from the results of basic kinetic studies to the crystal structures determined recently, is fairly complete (1,26,34,35). However, an interpretation of the enzymatic mechanism of this enzyme at molecular level has not been successfully obtained yet.

The techniques employed in this study, kinetics and NMR spectroscopy, are ideal for studying protein properties in solution. Using the crystal structure of phosphorylase as a starting point, supplemented by other information about this enzyme, the results here have enabled me to gain more insight about the catalytic process of phosphorylase.

In phosphorylase, the phosphoryl group of the enzyme-bound PLP has been found essential for the enzymatic activity (1,26). This study showed that PLP phosphate does not affect the binding of substrate, glucose-1-P, to its specific site on the protein and so that this phosphate group is unlikely to act as a proton donor and/or acceptor during catalysis (53). Results of the effects of different anions on phosphorylase reconstituted with pyridoxal are consistent with the hypothesis proposed by Withers et al. (50) that the phosphoryl group of PLP forms a trigonal-bipyramidal intermediate during catalysis.

Because the glucose moiety in phosphorylase reconstituted with pyridoxal(5')diphosphate (1)-α-D-glucose could be incorporated in the nonreducing ends of glycogen (50), this reconstituted enzyme was proposed to simulate the transition state of the native phosphorylase. However, the activation energy of the above reaction is similar to that of the reaction catalyzed by native phosphorylase, suggesting the interaction between the phosphoryl groups of glucose-1-P and PLP may not appreciably affect the activation energy of catalysis. Besides, in this study, I found that molybdate, which mimics a trigonal-bipyramidal intermediate, does not function as a real transition-state analogue in pyridoxal phosphorylase. It was also found that the change of the electrophilicity of the central atom of anions does not significantly affect their binding to pyridoxal reconstituted enzyme. Therefore, the formation of a trigonal-bipyramidal intermediate of PLP phosphate us unlikely to be the rate-limiting step of the catalysis of phosphorylase. In phosphorylase, the phosphate group of PLP interacts strongly with its surrounding basic amino acid sidechains (53). Therefore, the arrangement of these amino acid residues may affect the geometry of the phosphate group. Because

the protein structure around the PLP binding site may be changed during catalysis, the tetrahedral PLP phosphate may be thus distorted into a new geometry which can accommodate one more functional group, either one oxygen of the phosphate group of the enzyme-bound glucose-1-P or another amino acid side chain, and form a trigonal-bipyramidal structure. If the new functional group is the phosphate of glucose-1-P, this interaction may somehow hold the glucose-1-P in a conformation more accessible to the attack of the functional groups in the active site region to invoke the cleavage of the C_1-O bond in glucose-1-P. Another possibility is that the formation of a trigonal-bipyramidal PLP phosphate is merely a structural prerequisite for a well-organized active site.

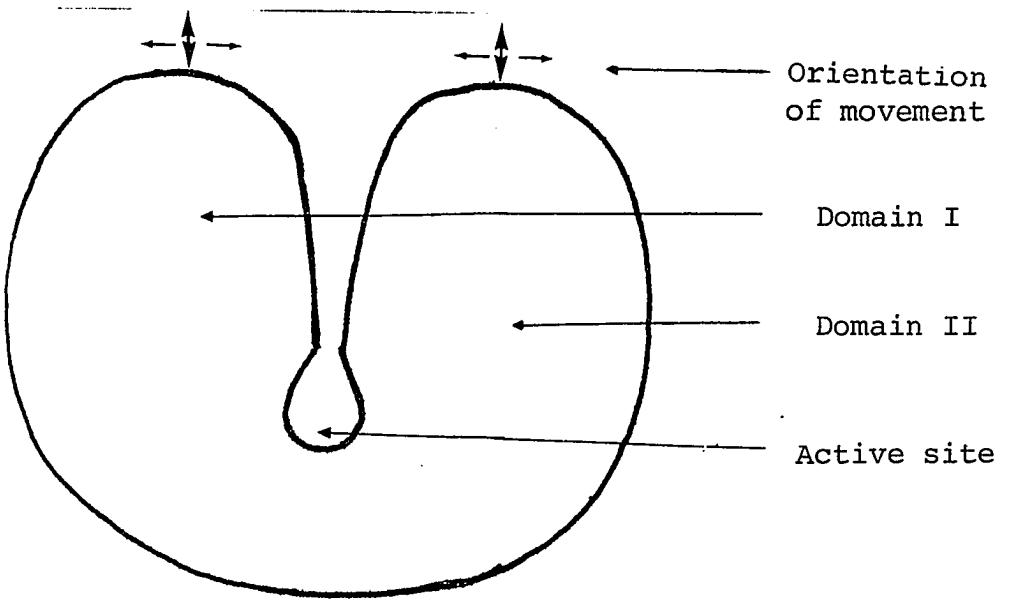
Results of kinetic, UV-fluorescence spectrophotometric, and NMR studies of phosphorylase reconstituted with 6-FPAL and 6-FPLP indicate that the pyridine part of the enzyme-bound PLP is likely to play an important structural role during the transformation of phosphorylase from an inactive to an activated state. In the activated state, the coenzyme molecule becomes less mobile, and its configuration is different from that in a "T" state enzyme. Besides, the 3-phenolic group of PLP is unlikely to be involved in an acid-base reaction during catalysis. The ring nitrogen may

interact with the protein, and this interaction could be important to the efficiency of catalysis. It is likely that the conformational changes at different parts of the protein, induced by the binding of ligands to their specific sites, can first affect the orientation of the pyridine ring of PLP. These conformational changes, then, could cause the alternation of the binding of the phosphate group in the active site region. The phosphate group can then hold the amino acid residues and/or the phosphate group in the right orientation for efficient catalysis.

Two interesting questions can be posed. First, what is the structural role fulfilled by pyridoxal phosphate in phosphorylase? Secondly, how can the binding of small molecule, such as glucose, glucose cyclic 1,2-phosphate, or AMP, induce pronounced structural changes of a large and complex molecule like phosphorylase? These questions may be partly answered if we assume that the structure of phosphorylase in solution is rather flexible than static as shown by X-ray crystallographic picture.

Peticolas (54) has pointed out that in solution the protein structure may be flexible and could undergo radial pulsation, torsional, twisted, shearing, or hinge-bending modes of movement. The characteristic low-frequency vibration of proteins may be due to these movements. Because the

picture obtained with X-ray crystallography is the molecule constrained by lattice force and averaged over disorder and thermal motions, the information of the internal motions and flexibility of phosphorylase is obscure. However, by examining the crystal structure of phosphorylase, it seems likely that the conformation of this protein may undergo certain motions in solution. The crystal structure of phosphorylase showed that the glucose-1-P binding site, presumably the active site, is buried at the heart of the molecule in the unique region where three domains come together (36), which is a common feature of enzymes (55). In phosphorylase, the active site is only accessible to the solvent through a narrow channel, about 15Å long. Therefore, a dynamic process closing and opening this channel must be involved in the binding of the substrates, glucose-1-P and glycogen. This dynamic process may be achieved by a certain relative movement of the domains of phosphorylase, as the hinge-bending mode suggested by Morgan and Peticolas (56), shown in the following figure.



The movement of each individual domain may be due to that when many atoms are coupled in the same domain, they can move collectively like small molecules in a crystal. Because each domain has its own pattern of motion (57), the motion of the whole protein, which is the combination of the motions of different domains, may have several different modes. Probably, only one of these modes meets all the requirements for carrying out catalysis, while the other modes produce structures that are enzymatically inactive. The distribution of different modes may be determined by their potential energies; the modes with higher energy are present in smaller proportions. The binding of different ligands to the protein may slightly modify the fine structure of the domains and thus affect the potential energies of

different modes. Therefore, the distribution of the enzyme in different modes may be changed as shown by the change of enzymatic activity. Because PLP resides right next to the active site and the structural transformation of phosphorylase from an inactive to an active state can cause significant changes both on PLP binding site and the coenzyme itself, it is likely that PLP in phosphorylase is interacting with different domains and hence coordinates the motions of different domains of the protein. Thus, the function of PLP in phosphorylase may be in keeping the internal motions of the protein in right modes. In addition, that the phosphoryl group of PLP may participate in the catalytic process of phosphorylase can not, however, be ruled out.

So far, our understanding of the catalytic process of glycogen phosphorylase is not complete. It might be improved by the following experiments. First, different glucose-1-P analogues could be synthesized chemically in order to find a real transition-state analogue, which should bind to the enzyme at least $10^4 \sim 10^5$ times more tightly than the normal substrate (58, 59), or to find a substrate analogue which can freeze the enzyme in the fully activated state long enough to be studied by physical or chemical methods. The second experiment is to continue the X-ray crystallographic study of phosphorylase crystals at the fully activated

state. Thirdly, different reporting groups, such as ^{13}C, ^{2}H, or ^{19}F could be incorporated at different parts of the protein. By monitoring their relative position, that whether the crystal structure determined by X-ray crystallography is still maintained in solution could be tested. Finally, the low-frequency vibration of phosphorylase could be studied by using Raman spectroscopy as to find the relationship between the enzymatic activity with the protein movement. With these results we may have better understanding of the catalytic process of phosphorylase at the molecular level.

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Yen